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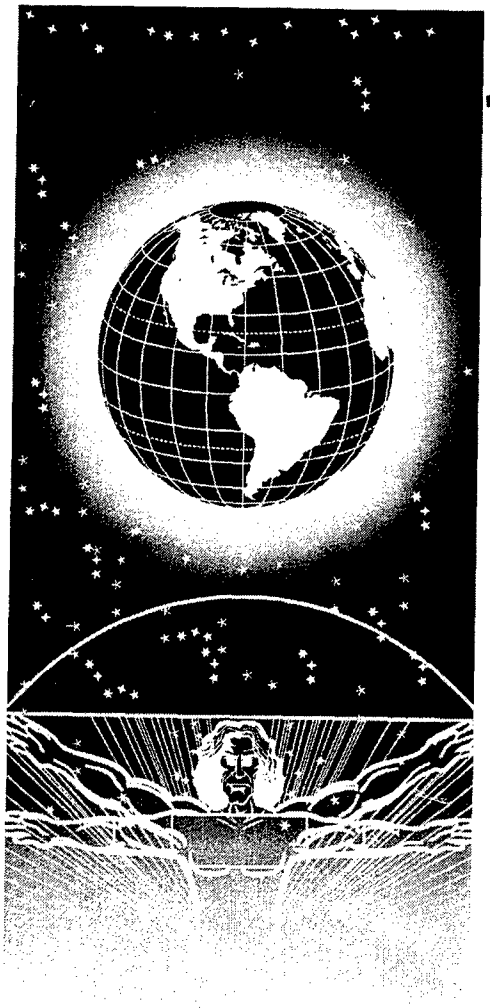

UNITED STATES AIR FORCE RESEARCH LABORATORY

1997 TOXIC HAZARDS RESEARCH ANNUAL REPORT

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MANTECH-GEO CENTERS JOINT VENTURE
PO BOX 31009
DAYTON, OH 45437-0009

May 1998
Interim Report - October 1996 - September 1997

Human Effectiveness Directorate
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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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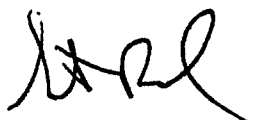

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13. ABSTRACT *(Maximum 200 words)*

This report is the 34th Annual Report of the Toxic Hazards Research (THR) program and presents scientific activities of the ManTech Geo-Centers Joint Venture contract on behalf of the toxicology divisions/detachments of the Air Force, Army, and Navy for the period of 1 October 1996 through 30 September 1997. The THR program conducts descriptive, mechanistic, and predictive toxicology studies and toxicological risk assessments to provide data to predict health hazards and to assess the health risks associated with human exposure to chemicals and chemical materials of interest to the military. The major goal of the THR's research efforts is to contribute to safe military operations, including safe occupational and environmental conditions. An additional goal of THR is to advance the state-of-the-art in toxicology research and risk assessment techniques. The scientific sections of the report are divided into the following six projects: trichloroethylene carcinogenicity; halon replacement toxicity; explosives, propellants, fuels, and lubricants; predictive toxicology; advanced composite materials; and methods development. Presented, also, are sections on conference support and research support (e.g., pathology, quality assurance, computer support) activities, as well as a products list of publications, presentations, and awards.

14. SUBJECT TERMS
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PREFACE

The 34th Annual Report of the Toxic Hazards Research (THR) program presents research and research support efforts conducted jointly by ManTech Environmental Technology, Inc. and Geo-Centers, Inc. on behalf of the U.S. Air Force, the U.S. Army, and the U.S. Navy under Department of the Air Force Contract No. F41624-96-C-9010. This document represents the second annual report for the current THR contract and describes accomplishments from 01 October 1996 through 30 September 1997.

Operation of the THR program under the current contract was initiated on 16 January 1996, under Project No. 7757, "Occupational and Environmental Toxic Hazards in Air Force Operations," Work Unit No. 7757A002, "Toxic Hazards Research." This research effort is cosponsored by the Army Medical Research Detachment, Walter Reed Army Institute of Research (WRAIR), Work Unit Nos. 611102.S15L and 612787.878L, and by the Naval Medical Research Institute Detachment/Toxicology (NMRI/TD), Work Unit No. M0096.004.0006, "Criteria for Exposure Limits in Navy Operational Environments."

The Toxicology Division, Occupational and Environmental Health Directorate, Armstrong Laboratory (AL/OET), Wright-Patterson Air Force Base, Ohio, provided the technical direction for this contract. Lt Col Terry A. Childress, Director of the Toxicology Division, served as the Contract Officer's Representative. The portion of the work effort sponsored by the Army was under the direction of LTC Roland E. Langford, Detachment Commander of the Medical Research Detachment. The portion of the work effort sponsored by the Navy was under the direction of the NMRI/TD Officer-in-Charge, CAPT Kenneth R. Still, MSC, USN. Darol E. Dodd, Ph.D., served as the ManTech Geo-Centers Joint Venture Program Manager for the THR program.

The contents and the preparation of this report represent the combined effort of the THR's ManTech Geo-Centers Joint Venture staff. Acknowledgment is made to Ms. Susie Godfrey and Ms. Teresa Ellis for their assistance in the preparation of this report.

ABBREVIATIONS

ABBREVIATIONS

1,2-DCVC	S-(1,2-Dichlorovinyl)-L-cysteine
2,2-DCVC	S-(2,2-Dichlorovinyl)-L-cysteine
AFMC	Air Force Materiel Command
BSP	Bromosulfophthalein
BSP-dCys	BSP dicysteine
BSP-dGSH	BSP diglutathione
BSP-mCys	BSP monocysteine
BSP-mCys-Gly	BSP monocysteinylglycine
BSP-mGSH	BSP monoglutathione
CH	Chloral hydrate
cm	Centimeter
CV	Coefficient of variation
d3-N-Ac-1,2-DCVC	[d3-Acetyl]N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine
DBN	1,5-Diazabicyclo[4.3.0]non-5-ene
DCA	Dichloroacetic acid
DNA	Deoxyribonucleic acid
EM	Electron microscope/microscopy
ESI	Electrospray ionization
ESI/MS	Tandem electrospray mass spectrometry
eV	Electron volt
g	Gram
G. Pig	Guinea Pig
GC	Gas chromatograph
GC/MS	Gas chromatograph mass spectrometry
h	Hour
HM	Hazardous material
HPLC	High performance liquid chromatopraphy
HW	Hazardous waste
kV	Kilovolt
m/z	Mass to charge ratio
mA	Microampere
mg	Microgram
min	Minute
mL	Microliter
mL	Milliliter
mm	Millimeter
MS	Mass spectrometry
N-Ac-1,2-DCVC	N-Acetyl-S-(1,2-dichlorovinyl)-L-cysteine
N-Ac-2,2-DCVC	N-Acetyl-S-(2,2-dichlorovinyl)-L-cysteine
ng	Nanogram
nl	Neutral loss
nm	Nanometer
ppm	Parts per million
psi	Pounds per square inch

(Abbreviations...continued)

RNA	Ribonucleic acid
s	Second
S/N	Signal to noise ratio
SEM	Scanning electron microscope/microscopy
SIM	Selective ion monitoring
SRM	Selective reaction monitoring
TCA	Trichloroacetic acid
TCE	Trichloroethylene
TCOG	Trichloroethanol glucuronide
TCOH	Trichloroethanol
TEA	Triethylamine
TEM	Transmission electron microscope/microscopy
TFA	Trifluoroacetic acid

SECTION 1

INTRODUCTION

INTRODUCTION

D.E. Dodd

This report presents a review of the activities of the ManTech Geo-Centers Joint Venture Toxic Hazards Research (THR) program, for the period 01 October 1996 through 30 September 1997. The THR program is an on-site, contractor-operated, United States Air Force, Army, and Navy multidisciplinary research operation. The THR program conducts descriptive, mechanistic, and predictive toxicology studies and toxicological risk assessments to provide data to predict health hazards and to assess the health risks associated with human exposure to chemicals and chemical materials of interest to the military. The major goal of the THR program is to contribute to safe military operations, including safe occupational and environmental conditions. An additional goal of THR is to advance the state-of-the-art in toxicology research and risk assessment techniques.

The THR program conducts research on a variety of materials that may range from pure chemicals to poorly defined mixtures. They include, but are not limited to fuels, lubricants, solvents, additives, components of explosives, propellants, paints, solvents, structural materials, training agents, and combustion products. Descriptive toxicology is used to identify toxic effects, target organs, and dose-response effects associated with different exposure routes, concentrations, and duration. Mechanistic toxicology is performed to determine toxicokinetics, mechanisms of action, and dynamics of expression of the toxic effects of the material of interest. Predictive toxicology involves the development, validation, and application of computer simulation models to describe quantitative dose-response relationships based on quantified input parameters such as exposure concentration, partition coefficients, respiratory rate, blood flows, rate of metabolite formation, rate of chemical excretion, and metabolic enzyme constants. These models are used to define target organ toxicity based on the tissue-specific dose and are used in intra- and inter-species extrapolation. Data generated via descriptive, mechanistic, and predictive toxicology studies are used together with interpreted literature data to produce human health hazard risk assessments.

In accordance with the THR contract's Statement of Work and specific technical directives (project requests) provided by the Contract Officer Representative, the THR staff also coordinates toxicology conferences, expert workshops, and program reviews. Research support benefiting both THR and government research efforts is provided in the areas of special test equipment design, fabrication, validation, modification, and maintenance; mathematics and biometry; computer systems management and programming; necropsy and histology techniques; management of toxicology information databases; quality assurance; health and safety; and documentation and report preparation. The THR program's research support and administrative elements are integral to the quality, continuity, and productivity of its scientific research efforts.

The research and support efforts of the THR program represent a continuum of activities that may overlap two or more years depending upon the study scheduling and the extent of the research that is required. During this reporting period, studies performed in response to requirements of the Air Force, Navy, and Army were categorized into six technical areas and two support areas. The six technical areas were the trichloroethylene (TCE) carcinogenicity project; the halon replacement toxicity project; the explosives, propellants, fuels, and lubricants project; the predictive toxicology project; the advanced composite materials project; and methods development. Research support areas included conference support and general (pathology, quality assurance, computer management) support. A number of technical support efforts in animal necropsy, histology, and equipment design and fabrication were conducted.

During this reporting period, the THR program provided work effort in support of toxicology conferences and workshops, including the series of annual toxicology conferences that have been coordinated by the THR unit (THRU) since 1965. The proceedings of the 1996 toxicology conference, "Conference on Advances in Toxicology and Applications to Risk Assessment" were compiled by the THR staff and distributed as a special technical report (AL/OE-SR-1997-0009). Selected publications were published in *Journal of Toxicology and Environmental Health.* The 1997 toxicology conference "Conference on Issues and Applications in Toxicology and Risk Assessment" was conducted 7-10 April at the Hope Hotel and Conference Center at Wright-Patterson Air Force Base (WPAFB). The proceedings of this conference were compiled by Teresa Ellis and published as a special issue in *Drug and Chemical Toxicology*, volume 20(4), 1997. In August 1997, the THR program initiated its planning efforts for the 1998 toxicology conference.

The execution of the THR contract's Statement of Work involves the integrated effort of a multidisciplinary staff of scientists, research technicians, and administrative and research support personnel. Sections 3 through 8 of this report emphasize the technical activities of the THR program. Sections 9 and 10 present highlights of the conferences and research support activities. Section 11 of this report is a set of appendices that describe the THR organization, its personnel, and its awards, publications, and presentations.

Historically, the THR program, *aka* THRU, has prepared annual reports on its research efforts since 1963. In general, these annual reports present summaries or highlights of the technical projects (project requests) that were directed by the Air Force, Army, and Navy. More descriptive reports on THR's research activities are prepared upon completion of project requests and are published as technical reports or peer-review publications (refer to "Products List for 1996-1997" in Section 11). Technical reports are also prepared following the conferences and most workshops coordinated by the THR staff. Copies of these technical reports are available from the National Technical Information Service or the Defense Technical Information Center.

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JOINT VENTURE STAFF
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SECTION 3

TRICHLOROETHYLENE (TCE) CARCINOGENICITY PROJECT

3.1 SYNTHESIS AND ANALYSIS OF MERCAPTURIC ACID METABOLITES OF TRICHLOROETHYLENE

W.T. Brashear and C.T. Bishop[1]

ABSTRACT

Trichloroethylene (TCE) is a commonly used industrial solvent which has been identified as a widespread groundwater contaminant. Because of the production of toxic metabolites, the metabolism of TCE is important. TCE may be metabolized by two different pathways. One route is metabolism by cytochrome P450, this results in the production of carboxylic acid metabolites, trichloroacetic acid (TCA) and dichloroacetic acid (DCA), which produce tumors in $B_6C_3F_1$ mice. A second metabolic pathway involves conjugation by glutathione S-transferase to yield *S*-(1,2-dichlorovinyl)glutathione, and *S*-(2,2-dichlorovinyl)glutathione. The glutathione conjugates can undergo further transformation to mercapturic acid which are nephrotoxic and have been implicated in renal tumorigenicity.

Urine samples from human volunteers were analyzed for the mercapturic acid metabolites of TCE. Eight subjects were exposed to 100 ppm TCE for 4 h. Urine samples were collected for 72 h following exposure. Since the mercapturic acid metabolites of TCE are not commercially available, these compounds were synthesized from TCE and L-cysteine. The products were purified by preparative HPLC, and their identity confirmed by GC/MS. Urine samples were analyzed for *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine, and *N*-acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine by GC/MS with selected ion monitoring. The deuterated analog d_3-*N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine was used as an internal standard. The limit of detection was 0.05 µg/mL in urine. Mercapturic acid metabolites of TCE were not found at this level of sensitivity.

INTRODUCTION

Trichloroethylene (TCE), a widely used degreasing and cleaning solvent, is an environmental contaminant found in groundwater. Exposure to TCE is of concern because it has been found to be a rodent carcinogen (Bruckner et al., 1989). TCE can undergo oxidative metabolism and may also be conjugated with glutathione. When oxidized by cytochrome P450, TCE is metabolized to chloral hydrate (CH), trichloroacetic acid (TCA), dichloroacetic acid (DCA), trichloroethanol (TCOH), and trichloroethanol glucuronide (TCOG) (Tanaka and Ikeda, 1968; Hathway, 1980). DCA and TCA, like TCE, cause mouse liver tumors (Bull et al., 1990; DeAngelo et al., 1991). In contrast to mouse, TCE is nephrotoxic in rat (Goeptar et al., 1995). Nephrotoxicity results from the conjugation of TCE with glutathione. As shown in Figure 3.1-1, TCE can react with the cysteinyl sulfhdryl group of reduced glutathione (McKinney et al., 1959).

[1]Air Force Research Laboratory, Operational Toxicology Branch, Wright-Patterson Air Force Base, OH.

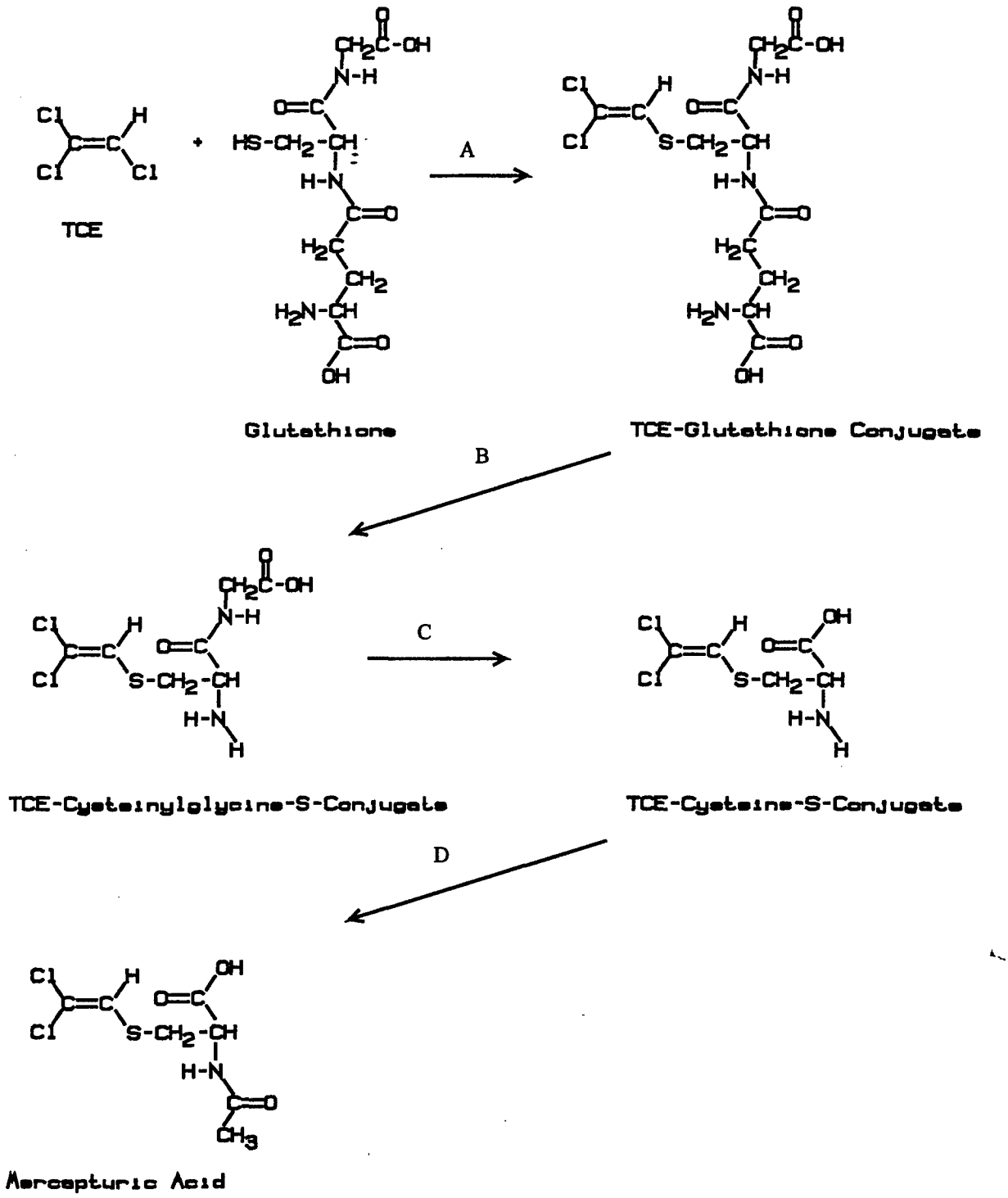

Figure 3.1-1. Metabolism of TCE to mercapturic acid. Reactions catalyzed by: A) glutathione-S-transferase, B) γ-glutamyltransferse, C) cysteinylglycine dipeptidase, D) L-cysteine *S*-conjugate *N*-acetyltransferase.

The glutathione conjugate of TCE undergoes proteolysis first by γ-glutamyltransferase, and then cysteinylglycine dipeptidase to yield the cysteine conjugate of TCE. The cysteine conjugate can be N-acylated by L-cysteine *S*-conjugate *N*-acetyltransferase to form a mercapturic acid.

Male rats exposed to TCE have shown an increased incidence in kidney tumors and toxic nephrosis (Commandeur and Vermeulen, 1990). Nephrotoxicity has been associated with small amounts of urinary mercapturic acids. The cysteine conjugate, *S*-(1,2-dichlorovinyl)-L-cysteine (1,2-DCVC) has been shown to be mutagenic (Commandeur and Vermeulen, 1990). Studies with ^{35}S cysteine conjugates have shown they are rapidly metabolized, and incorporated into the tissue of rat kidney proximal tubules. When the ^{35}S cysteine conjugates are N-acylated to mercapturic acids, they are not metabolized and incorporated into tissue (Zhang and Stevens, 1989). This suggests that mercapturic acids need to be deacetylated to a cysteine conjugate in order to be cytotoxic.

The cysteine conjugates of TCE are intermediate metabolites which can be bioactivated by L-cysteine *S*-conjugate β-lyase (Goeptar et al., 1995). This enzyme cleaves the C-S bond of the TCE cysteine conjugate producing pyruvate, ammonia, and an enethiol. The action of β-lyase on 1,2-dichlorovinyl-*S*-cysteine forms an vicinal[2] dichloro enethiol which can isomerize to form thionoacyl chloride, or can eliminate HCl to yield a highly reactive thioketene. Both thionoacyl chloride and thioketene are highly reactive thioacylating agents (Goeptar et al., 1995). In contrast, the action of β-lyase on the 2,2-dichlorovinyl-*S*-cysteine isomer leads to the formation of an geminal[3] dichloro enethiol which can isomerize to thioaldehyde. This compound is not a direct alkylating agent. The higher mutagenicity of 1,2-dichlorovinyl-*S*-cysteine, relative to 2,2-dichlorovinyl-*S*-cysteine, is consistent with the higher reactivity of the β-lyase products of 1,2-dichlorovinyl-*S*-cysteine. The β-lyase reactions of the TCE cysteine conjugates are shown in Figure 3.1-2.

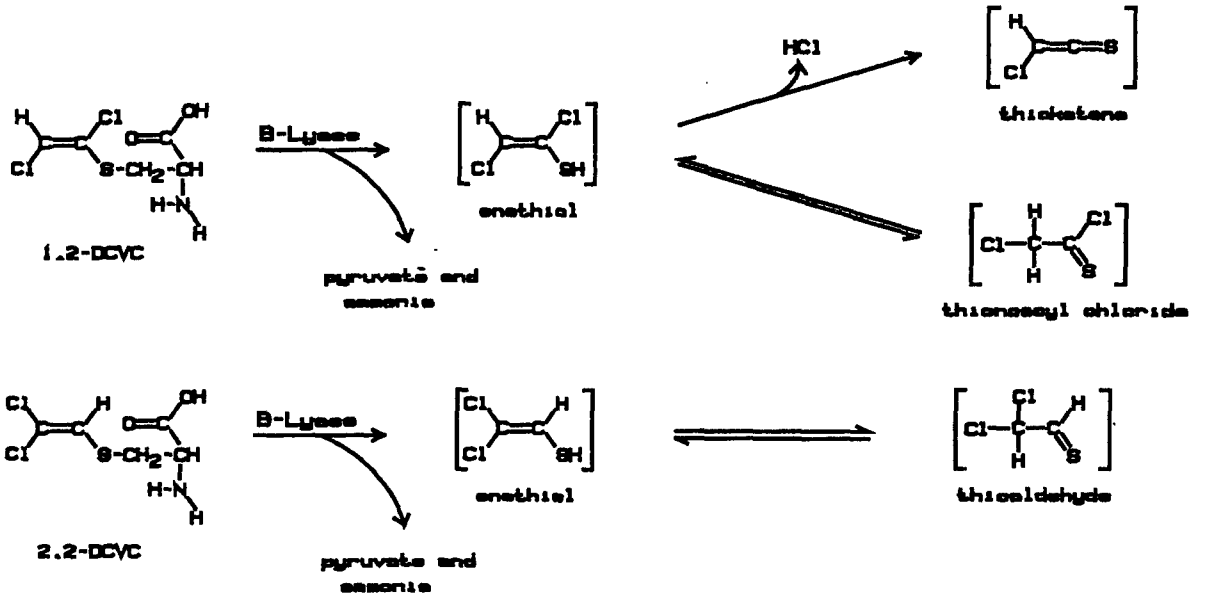

Figure 3.1-2. **Metabolism of TCE cysteine conjugates by β-lyase: 1,2-Dichlorovinyl-*S*-cysteine (1,2-DCVC) forms a vicinal enethiol which can eliminate HCl to yield a highly reactive thioketene, or rearrange to a highly reactive thioacyl chloride. 2,2-Dichlorovinyl-*S*-cysteine (2,2-DCVC) forms a geminal enethiol which rearranges to dichlorothioaldehyde.**

[2] Chlorine atoms are located on adjacent carbon atoms.
[3] Chlorine atoms are located on the same carbon atoms.

The purpose of this study was to prepare analytical standards of the mercapturic acid metabolites of TCE and to use them for the analysis of urine samples. The urine samples were obtained from human volunteers who where exposed to 100 ppm TCE for 4 h. Urine samples were analyzed for 1,2-dichlorovinyl-*S*-cysteine and 2,2-dichlorovinyl-*S*-cysteine by combined GC/MS with selective ion monitoring.

MATERIAL & METHODS

Chemicals & Equipment

TCE was obtained from Aldrich Chemical Company (Milwaukee, WI). Trifluoroacetic acid (TFA), L-Cysteine, *N*-acetyl-L-cysteine, sodium, anhydrous ammonia, 1,5-diazabicyclo[4.3.0]non-5-ene, acetic anhydride, and d_6-acetic anhydride were also obtained from Aldrich Chemical Company. Sulfuric acid, acetic acid, and ethyl acetate were obtained from Fisher Scientific (Fair Lawn, NJ). Diethyl ether was obtained from Baxter Healthcare Corporation, Burdick & Jackson Division (Muskegon, MI). A Thermolyne model 16700 vortex mixer was used to vortex and extract samples (Dubuque, IA). An N-Evap analytical evaporator from Organomation Inc. (Berlin, MA) was used to evaporate ether extracts. Samples were analyzed on a

Hewlett-Packard 5971 Mass Selective Detector and a Hewlett-Packard 5890 Series II Gas Chromatograph, equipped with a 30m x 0.25mm HP-5 column (Hewlett-Packard, Avondale, PA). High performance liquid chromatography was done using an LDC 4100 MS solvent delivery system, an LDC 4100 autosampler, and an LDC 5000 UV monitor (Thermo Instrument Systems Inc., Riviera Beach, FL). Separation was done on a Supelcosil, 4.6mm x 25cm C-18 analytical column, and 1 x 25cm^2 Supelcosil 25 C-18 preparative column. Both were obtained from Supleco Chromatography Products (Bellefonte, PA).

Assay Procedure

TCE Exposure

Human volunteers were exposed to 100 ppm TCE via inhalation for 4 h. A baseline urine sample was collected prior to exposure. Urine samples were collected after the exposure at t = 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 30, 44, 50, 55, and 72 h. The urine samples were refrigerated immediately after collection, and frozen at -20 °C prior to analysis. Sample sets from 8 volunteers were analyzed for *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine (*N*-Ac-1,2-DCVC) and *N*-acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine (*N*-Ac-2,2-DCVC) by combined GC/MS.

Synthesis of Standards

The regioisomers, *N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine and *N*-acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine were synthesized as mercapturic acid standards. The deuterated analog of *N*-Ac-1,2-DCVC, [d_3-acetyl]*N*-acetyl-*S*-(1,2-dichlorovinyl)-L-cysteine (d_3-*N*-Ac-1,2-DCVC), was synthesized as an internal standard.

The standard *N*-Ac-1,2-DCVC was synthesized in a two step procedure. Initially, the cysteine conjugate of TCE, *S*-(1,2-dichlorovinyl)-L-cysteine (1,2-DCVC), was synthesized from TCE, and L-cysteine in liquid ammonia with sodium by the procedure of McKinney (McKinney et al., 1959). This is shown in step A of Figure 3. The product, 1,2-DCVC, was then acetylated with acetic anhydride to yield *N*-Ac-1,2-DCVC (Birner et al., 1993) (step B). The internal standard, d_3-*N*-Ac-1,2-DCVC, was synthesized in the same manner by reacting 1,2-DCVC with d_6-acetic anhydride (step C). The regioisomer, *N*-acetyl-*S*-(2,2-dichlorovinyl)-L-cysteine (*N*-Ac-2,2-DCVC), was synthesized by adding TCE to *N*-acetyl-L-cysteine, and 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) in dimethylsulfoxide (DMSO) according to the procedure of Birner and Dekant (Birner et al., 1996) (step D). The synthetic reactions are summarized in Figure 3.1-3.

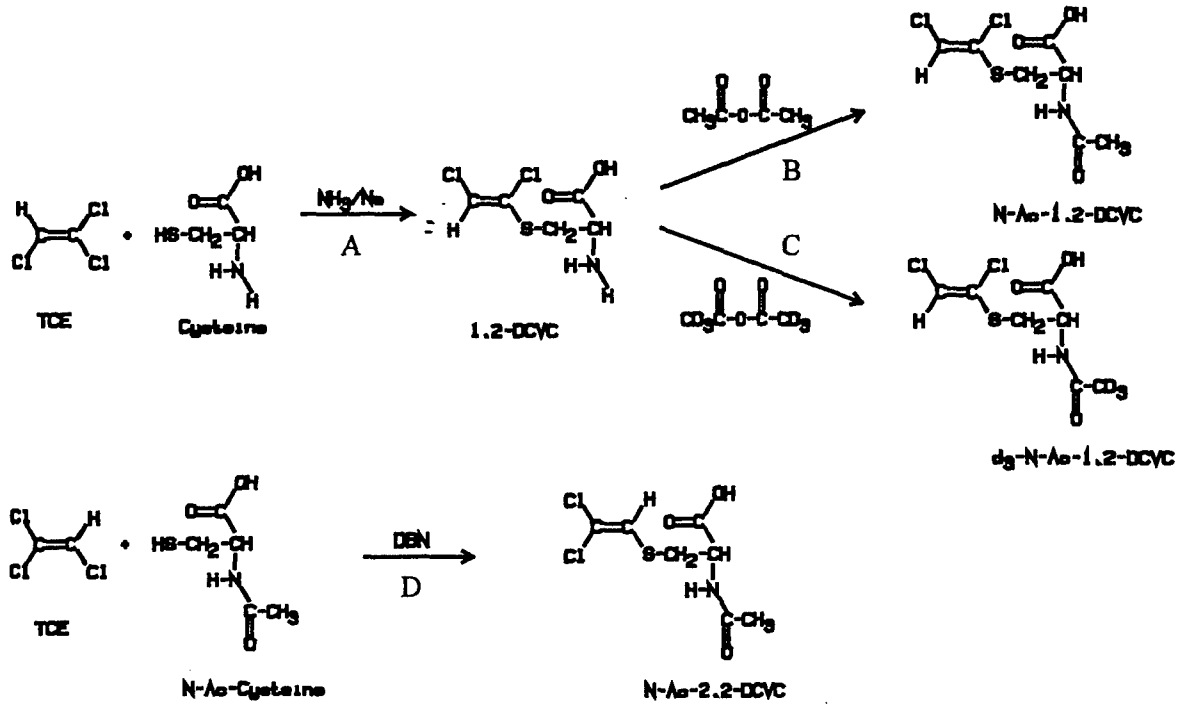

Figure 3.1-3. **Synthetic reactions for mercapturic acids and internal standard: A) TCE and cysteine combined with sodium in liquid ammonia to form 1,2-DCVC. B) 1,2-DCVC reacted with acetic anhydride to form *N*-Ac-1,2-DCVC. C) 1,2-DCVC reacted with d_6-acetic anhydride to form d_3-*N*-Ac-1,2-DCVC. D). TCE reacted with *N*-acetyl cysteine in DMSO with DBN to form *N*-Ac-2,2-DCVC.**

The products from the synthetic reaction mixtures were purified by preparative HPLC, using a 1 x 25cm C-18 column (Supelcosil). The column was eluted at 4 mL/min with 40% methanol, 60% 0.1% TFA. The UV absorbance was monitored at 225 nm.

Extraction Procedure

The extraction and analysis procedure was adapted from a method published by Birner et al. (Birner et al., 1993). Standard curve samples were prepared from control urine and 2 μg of internal standard (d_3-*N*-Ac-1,2-DCVC) was added to each sample. A 4 mL urine sample was acidified with 0.5 mL 10% sulfuric acid and extracted three times with 2 mL aliquots of diethyl ether. The combined ether extracts were concentrated to 1 mL and 0.1 mL of ethereal diazomethane was added (Aldrich Technical Bulletin AL-180, 1990). After 5 min, the ether was evaporated to dryness and reconstituted in 0.25 mL of ethyl acetate. A 1 μL aliquot was injected onto the gas chromatograph. The chromatography conditions are given in Table 3.1-1.

Table 3.1-1. Chromatography Conditions

Injector Temperature	175 °C
Initial Temperature	60 °C
Initial Time	2 min
Final Temperature	165 °C
Rate	20 °C/min
Final Time	22.75 min
Run Time	30 min

The ion at m/z 144 was monitored for *N*-Ac-1,2-DCVC and *N*-Ac-2,2-DCVC, which had retention times of 27.4 min, and 28.3 min, respectively. The ion at m/z 147 was monitored for the d_3-*N*-Ac-1,2-DCVC internal standard, which had a retention time of 27.3 min. A first order polynomial without weighting was used to fit standard curves which ranged from 10 ng/mL to 100 ng/mL.

Results

The synthesis and preparative HPLC purification of *N*-Ac-1,2-DCVC and *N*-Ac-2,2-DCVC gave compounds which were 99+ % pure. The identity of these compounds was confirmed by GC/MS and the mass spectra of these compounds is shown in Figures 4 and 5, respectively. The mass spectra of *N*-Ac-1,2-DCVC and *N*-Ac-2,2-DCVC agreed with published literature spectra (Commandeur and Vermeulen, 1990).

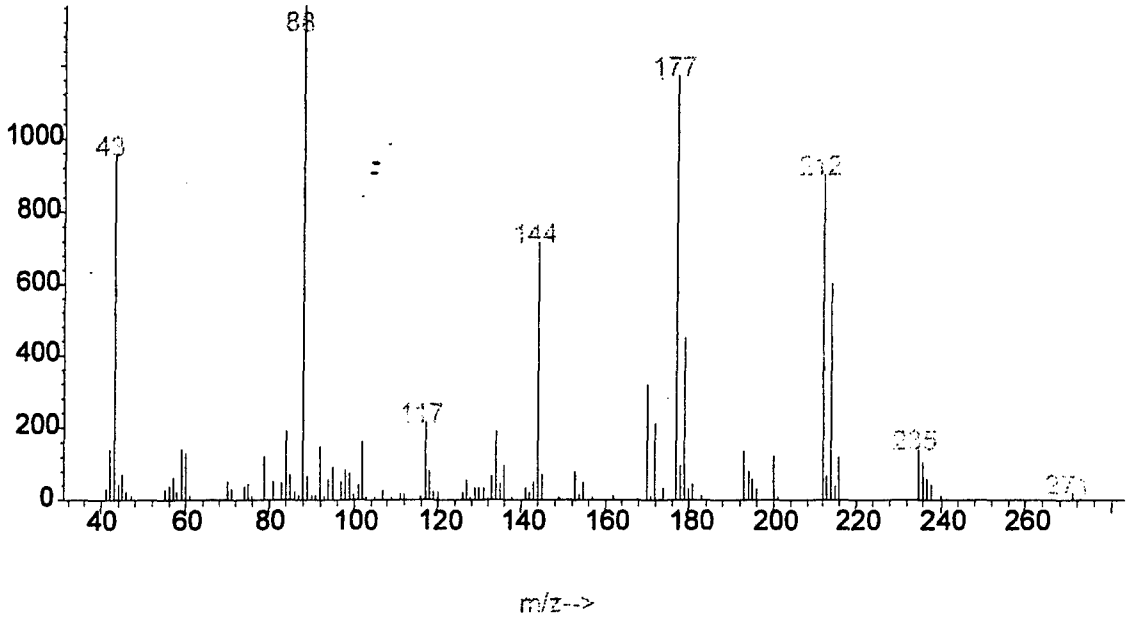

Figure 3.1-4. **Mass spectra of *N*-Ac-1,2-DCVC. M/z values are plotted against the ion abundance.**

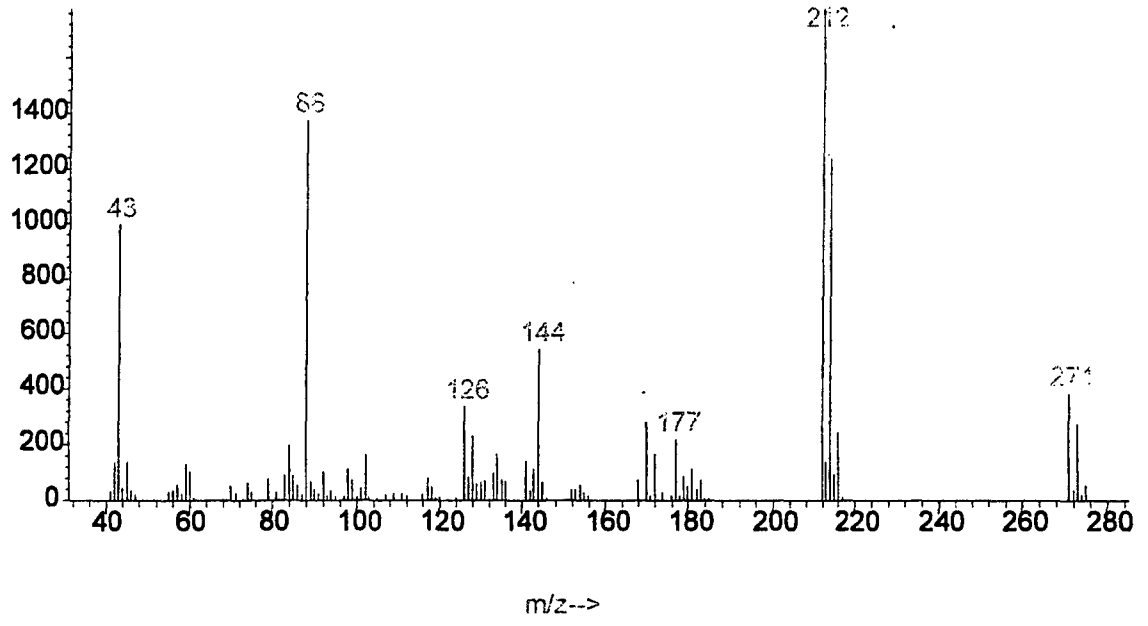

Figure 3.1-5. **Mass spectra of *N*-Ac-2,2-DCVC. M/z values are plotted against the ion abundance.**

Standard curves obtained from urine were linear up to 1000 ng/mL for *N*-Ac-1,2-DCVC and *N*-Ac-1,2-DCVC. The detection limit was 50 ng/mL (S/N > 3, 99.9 %). The ion-chromatograph from a 100 ng/mL standard of *N*-Ac-1,2-DCVC and *N*-Ac-2,2-DCVC in urine is shown in Figure 3.1-6.

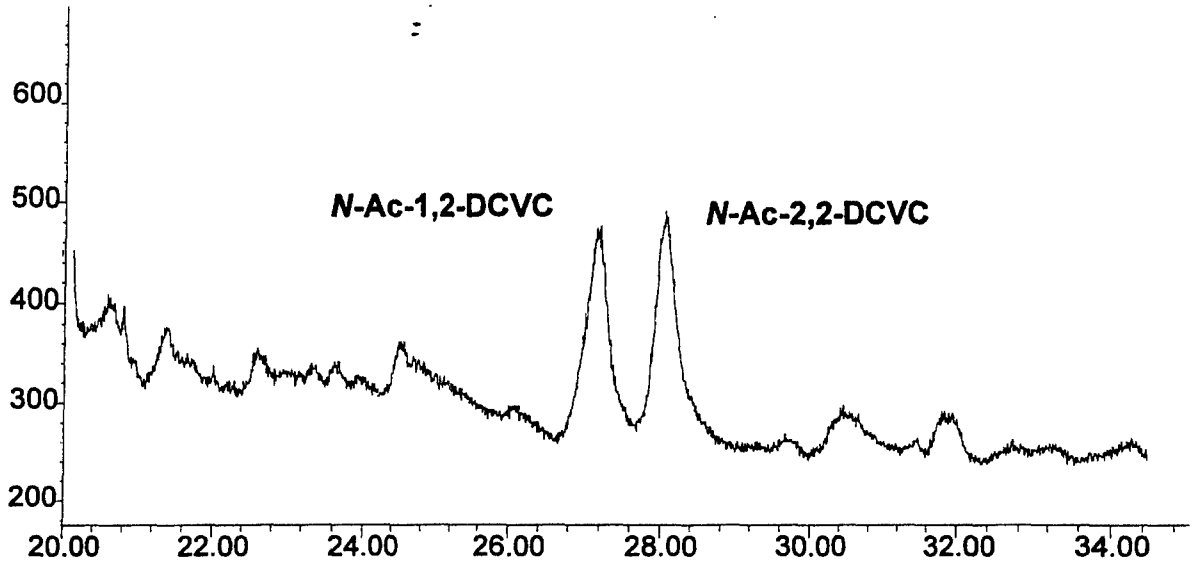

Figure 3.1-6. Extracted ion chromatogram at m/z 144 for *N*-Ac-1,2-DCVC, and *N*-Ac-2,2-DCVC in urine at 100 ng/mL. Retention time is plotted against ion abundance. *N*-Ac-1,2-DCVC had a retention time of 27.4 min, and *N*-Ac-2,2-DCVC had a retention time of 28.3 min.

The extraction efficiency of *N*-Ac-1,2-DCVC and *N*-Ac-2,2-DCVC from urine was 75%. The reaction efficiency of the diazomethane derivatization was 90%. Typical curves for *N*-Ac-1,2-DCVC and *N*-Ac-2,2-DCVC were: $y=1.790E\text{-}3x+0.0840$, $r^2>0.995$; and $y=3.16E\text{-}3x+0.022$, $r^2>0.999$, respectively.

The precision and accuracy of the method was determined. The intra-day variability is shown in Table 3.1-2. *N*-Ac-1,2-DCVC and *N*-Ac-2,2-DCVC were extracted and analyzed from urine at low, medium, and high concentrations. The precision samples for intra-day variability gave results which were within 95% of their expected values. The coefficient of variation averaged 29% for *N*-Ac-1,2-DCVC and 25% for *N*-Ac-2,2-DCVC.

Urine samples from 4 volunteers exposed to 100 ppm TCE by inhalation for 4 h were analyzed for *N*-Ac-1,2-DCVCand *N*-Ac-2,2-DCVC. Samples taken up to 48 h after TCE exposure were analyzed. At a detection limit of 50 ng/mL, no mercapturic acids were found.

Table 3.1-2. Intra-day Variability for Mercapturic Acid Metabolites as Determined by GC/MS

	Actual Concentration ng/mL	Number of Replicates	Accuracy (mean % found/added)	Precision (% CV)
N-Ac-1,2-DCVC				
Intra-day	**50**	**4**	**98**	**40**
	100	**8**	**101**	**21**
	500	**4**	**92**	**25**
N-Ac-2,2-DCVC				
Intra-day	**50**	**4**	**100**	**21**
	100	**8**	**93**	**31**
	500	**4**	**85**	**19**

DISCUSSION

This method uses GC/MS with electron impact ionization and selective ion monitoring to analyze urine samples for mercapturic acid metabolites of TCE. The assay has a limit of detection of 50 ng/mL for *N*-Ac-1,2-DCVC and *N*-Ac-2,2-DCVC. This is lower than the published detection limit of 3 μg/mL (Birner et al., 1993). The precision of the assay was evaluated at 50, 100, and 500 ng/mL. This gave results which agreed within 95% of the expected values, and had an average CV of 26%. The analysis of four sample sets of urine from volunteers exposed to 100 ppm TCE for 4 h did not contain any mercapturic acid metabolites. It is possible that these urine samples contain mercapturic acid metabolites below the limit of detection.

This possibility is strongly supported by a recent study by Bernauer et al., (Bernauer et al., 1996). In this study human urine samples were collected following TCE inhalation exposure. Test subjects were exposed to TCE at 40, 80, and 160 ppm for 6 h. This is similar to the dose used for our study. However, their urinary mercapturic acid levels were reported in [nmole/mg creatinine h]. This requires conversion to ng/mL to compare results. Assuming that approximately 1500 mg of creatinine is excreted in about 2500 mL of urine in 24 h, allows Bernauer's values to be expressed in ng/mL. This results in estimated peak mercapturic acid levels of 20 ng/mL for the 160 ppm exposure level, and 10 ng/mL for the 80 and 40 ppm exposures levels. These peak levels occurred at about 10 h postexposure. All of these levels are below our 50 ng/mL limit of detection. However, this analysis was performed by negative chemical ionization GC/MS with selected ion monitoring. This has a limit of detection below 1 ng/mL (Bernauer et al., 1996). Limits of detection this low cannot be achieved with GC/MS with electron impact ionization (Bernauer et al., 1996).

The 24 h urine volumes for our 4 test subjects averaged 2500 mL. However, one subject had a 24- h volume of only 1400 mL. This individual, who had more concentrated urine, also appeared to have mercapturic acid levels

which were just below the 50 ng/mL limit of detection. Specifically, this test subject had urinary *N*-Ac-2,2-DCVC at 10 and 12 h of 44 and 35 ng/mL, respectively. Although this is below the 50 ng/mL detection limit, these data may not be artifacts since they appear to be consistent with Bernauer's findings. Other subjects who had greater urine volume (approximately 3000 mL) did not have any mercapturic acids just below the dectection threshold. This suggests that mercapturic acids are present below the limit of detection. It is possible that the analysis of the urine samples by GC/MS with negative ion chemical ionization may yield mercapturic acid data.

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3.2 PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODELING OF OXIDATIVE METABOLISM OF TCE IN HUMANS: DISSECTION OF EXPERIMENTAL ERROR AND INTERINDIVIDUAL VARIABILITY

J.Z. Byczkowski and J.C. Lipscomb[1]

ABSTRACT

Isolated human hepatic microsomes are widely used as an *in vitro* experimental system to assess the metabolic fate of xenobiotics, decreasing the dependence upon data derived from animals. The microsomal systems *in vitro* were also applied to estimate metabolic clearance of trichloroethylene (TCE) in humans. In this report, we have focused on the extent of uncertainty that may be introduced into the mathematical modeling and risk assessment process by utilizing metabolism parameters measured *in vitro*. Initially, the following parameters were quantified for human liver in our laboratory: microsomal protein content and number of hepatocytes in liver, partitioning of TCE into microsomes, V_{max} and K_m in microsomes. Experimental error was estimated by repeated determinations of samples from the same individuals. The interindividual variability was estimated by determining average values for different individuals. Next, these parameters were extrapolated by a mathematical "drop-in" module to yield the input data for a physiologically based pharmacokinetic (PBPK) model which was used to simulate TCE distribution and metabolism in the human. Finally, a previously published PBPK model for TCE (Allen and Fisher, 1993), was modified by adding the "drop-in" module which incorporated recalculated TCE metabolism parameters (K_m and V_{max}) from the *in vitro* microsomal system, and by adding the Monte Carlo module which randomly sampled input values of parameters. This allowed us to run multiple PBPK simulations (within the experimentally determined range) with varied headspace TCE concentrations, microsomal protein contents, microsomal V_{max} and K_m, and the number of hepatocytes in the liver. A sensitivity analysis was conducted with this modified PBPK model to identify critical parameters and evaluate their impact on the model variables. Then, a Monte Carlo-type analysis was performed to visualize uncertainty propagation over time. The results were used to compare effects of the experimental error to those of the interindividual variability of cytochrome P-450 (CYP)-dependent metabolism of TCE. Our analyses have determined which measured CYP-dependent metabolism parameters contributed most to the overall uncertainty in the PBPK model output. As a result of this study, we conclude that experimental errors from an *in vitro* microsomal system added little to the uncertainty in the simulated values when compared with overwhelming differences in TCE metabolism in different human donors.

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INTRODUCTION

The increasing acceptance of computer-based pharmacokinetic modeling has resulted in the consideration of simulated data in human health risk assessments. One limitation of the modeling in humans is the necessity of model verification, usually done through human exposures. This may be impossible for intrinsically toxic compounds, but it has been accomplished for a relatively safe trichloroethylene (TCE; Allen and Fisher, 1993). Recently, metabolism parameters derived from *in vitro* measurements were used in the construction of a physiologically-based pharmacokinetic (PBPK) model for a structurally similar compound, perchloroethylene (Reitz et al., 1996). In the present study, we have applied a similar strategy, and have developed a "drop-in" mathematical module which can incorporate recalculated *in vitro* TCE metabolism parameters into the existing human PBPK model.

For TCE, a compound to which humans were safely exposed, the PBPK model can be calibrated and validated with both data from humans *in vivo* and *in vitro*. However, because the accuracy of the *in vitro/in vivo* extrapolation relies heavily on the quality of *in vitro* data, we first had to characterize the activity of CYP- dependent metabolism of TCE in human microsomes, basing our quantitative comparison on microsomal protein content in the liver, then we had to describe how the experimental error propagates along the model. Finally, we compared the overall uncertainty resulting from experimental measurements to that resulting from interindividual variability.

MATERIALS AND METHODS

Experimental data

The experimental data analyzed in this study were generated *in vitro* by Lipscomb et al. (1997) and compared to *in vivo* data from the exposed human subjects described by Muller et al. (1974, 1975) and Stewart et al. (1970).

Mathematical modeling

A physiologically based pharmacokinetic (PBPK) model was written in Advanced Continuous Simulation Language (ACSL, Mitchell and Gauthier, 1993), according to the mathematical description of Allen and Fisher (1993), with the addition of a "drop-in" module that recalculated metabolism parameters (V_{max} and K_m) from our *in vitro* experimental input data using the isolated human microsomes (for source codes see Appendix). Simulations were performed on a personal computer using the ACSL© 11.4 software (Mitchell and Gauthier Associates, Inc., Concord, MA) and the sensitivity analysis was conducted, essentially as described by Evans and Andersen (1995). The sensitivity coefficient was defined as a percent change in PBPK model output variable (OV) due to percent change in input parameter (IP):

$$\partial OV/\partial IP = [(OV_{affected} - OV_{default}) \times IP]/(OV_{default} \times \Delta IP)$$

Our input parameters, measured experimentally *in vitro*, were changed (ΔIP) either by 10% or by one experimental standard deviation (resulting from experimental errors).

For Monte Carlo analysis a separate module was developed according to Thomas et al. (1996). The Monte Carlo module was written in ACSL with some FORTRAN commands (as described by Thomas et al. 1996), and it randomly sampled input values of pre-determined critical experimental parameters, creating the master *.CMD file which was, in turn, used to execute the modified PBPK model. This allowed us to run multiple PBPK simulations (within the experimentally determined range) for varying headspace TCE concentrations, microsomal protein contents, Vmax and Km in microsomes, and the number of hepatocytes in the liver. The experimental input parameters from human hepatic microsomes (published previously by Lipscomb et al. 1997) were first evaluated statistically, and they seemed to follow a normal rather than a lognormal distribution with no apparent correlation between them. The means and standard deviations of the input parameters were either from the repetitive measurements of microsomal data for the same donor (to determine experimental error) or from a group of different donors (to determine an interidividual variability). The output of multiple computer simulations was directed to the graphic file for presentation (Figures 3.2-3 and 3.2-4).

RESULTS AND DISCUSSION

From fitting the human data for TCE exposure (Muller et al. 1974,1975) by the PBPK model, containing the "drop-in" module which converted our *in vitro* results into human metabolism parameters, it seems that our microsomal preparations resembled the TCE metabolic system of human subjects (Figure 3.2-1).

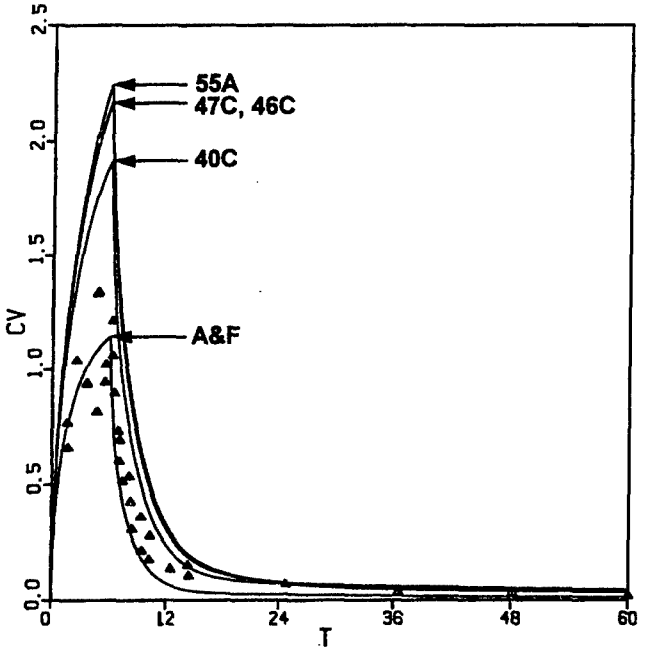

Figure 3.2-1. Comparison of fitting the human data for TCE concentration in blood after Muller et al., (1974 and 1975) by PBPK model using metabolism parameters determined *in vitro*: A&F - parameters optimized by Allen and Fisher (1993); 55A, 47C, 46C, 40C - parameters determined *in vitro* in isolated human liver microsomes (from donor #55A, #47C, #46C, and #40C, respectively; Lipscomb et al., 1997). Exposure scenario: single exposure to 100 ppm of TCE for 6 h T - time (hr); CV - venous blood TCE concentration (mg/l).

Given a tremendous difference in the structure and complexity between the *in vitro* and *in vivo* systems we were surprised to see the simulated peak venous blood concentration only twice greater than that optimized by Allen

and Fisher (1993). The difference by the factor of two may be due to our underestimated rate of microsomal metabolism of TCE and/or underestimated blood concentrations of TCE by Muller et al. (1974, 1975).

To demonstrate the usefulness of the microsomal *in vitro* data in reliably predicting the disposition of TCE in humans, we interfaced the PBPK model (equipped with a "drop-in" mathematical module) with a Monte Carlo module which randomly sampled input values of critical experimental parameters (Thomas et al., 1996). This allowed us to run multiple PBPK simulations (within the experimentally determined range) for varying headspace TCE concentrations, microsomal protein contents, V_{max} and K_m in microsomes, and the number of hepatocytes in the liver. At first, a sensitivity analysis was conducted to determine critical parameters and their impact on the model variables. Next, a Monte Carlo - type analysis was performed. The results were used to compare the experimental error to the interindividual variability of cytochrome P-450 (CYP)-dependent metabolism of TCE.

A sensitivity analysis, within the physiological modeling range, was performed to estimate how the *in vitro* experimental variability of the measured parameters may affect results of PBPK model simulation. Time of simulation for zero order parameters (V_{max} and K_m) was set at 2 h, whereas for time-dependent variables it was set at the maximum response (6 h for CV and AM in single exposure scenario according to Muller et al., 1974, and 103 h for CXppm in multiple exposure scenario according to Stewart et al., 1970). As presented in Table 3.2-1, the factors producing the greatest impact on final metabolism parameters estimated for human subjects were: the measurement of microsomal protein content (MPL), the determination of V_{max} in microsomes ($Vmax_M$) and the determination of Michaelis constant for microsomes (Km_M). The same *in vitro* measurements had less impact on simulated mixed venous blood concentration of TCE (CV) and on simulated amount of TCE metabolized (AM) in human subjects after single exposure (Muller et al., 1974), and had negligible impact on simulated concentration of exhaled TCE in human subjects after a multiple-exposure scenario (CXppm; Stewart *et al.*, 1970).

Because AM (amount of TCE metabolized in human subjects) is the PBPK model output variable which may likely drive the future risk assessments for TCE, we chose this variable to determine how sensitivity coefficients will propagate over time (Figure 3.2-2). Standard deviations, resulting from experimental error, for our *in vitro* experimental parameters in microsomal systems were determined and their impact on PBPK model simulated AM was plotted as a function of time. From results presented in Figure 3.2-2 it seems that experimental error resulting from measurements of V_{max} and protein concentration in the microsomal system had decreasing impact on the PBPK model-simulated TCE metabolism over time, while a very small impact of errors in K_m and the measurement of headspace TCE concentrations remained practically constant.

TABLE 3.2-1. BIOLOGICALLY RELEVANT SENSITIVITY ANALYSIS WITHIN THE PHYSIOLOGICAL MODELING RANGE: NORMALIZED RESPONSE OF PBPK MODEL TO 10% CHANGE IN THE INPUT VALUE OF *IN VITRO* PARAMETERS

Parameters from Microsomes
Sensitivity Coefficients:

Output variable	VMAX at 2 h
HDSC	0.059
MPL	1.00
$Vmax_M$	1.00
Km_M	-0.064
NHEP	0.000

Output variable	KM at 2 h
HDSC	0.000
MPL	0.000
$Vmax_M$	0.000
Km_M	1.01
NHEP	0.000

Output variable	CV at 6 h
HDSC	0.000
MPL	-0.204
$Vmax_M$	-0.204
Km_M	0.204
NHEP	0.000

Output variable	AM at 6 h
HDSC	0.041
MPL	0.691
$Vmax_M$	0.691
Km_M	-0.585
NHEP	-0.001

Output variable	CXppm at 103 h
HDSC	-0.006
MPL	-0.107
$Vmax_M$	-0.107
Km_M	0.073
NHEP	0.000

HDSC - headspace TCE concentration (5000 ppm);
MPL - microsomal protein content (20.8 mg/g liver);
$Vmax_M$ - Vmax in microsomes (1589 pmol/min/mg);
Km_M - Km for microsomes (399 ppm);
NHEP - number of hepatocytes in liver tissue (116 x 10^6/g liver);
VMAX - Vmax in human subject (mg/hr);
KM - Km in venous blood leaving human liver (mg/L);
CV - concentration of TCE in mixed venous blood (mg/L);
AM - amount of TCE metabolized by human subject (mg);
CXppm concentration of TCE in the exhaled air (ppm).

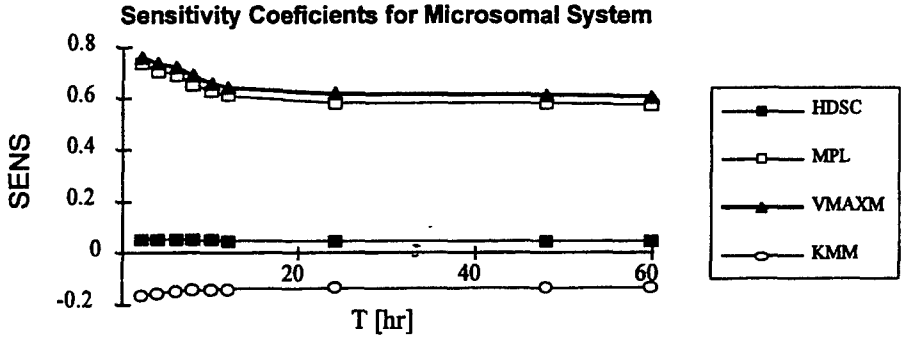

Figure 3.2-2. **Normalized sensitivity coefficients (SENS) with respect to amount of TCE metabolized (AM) as a function of time under single exposure scenario (Muller et al., 1974 and 1975). The following input parameters, measured experimentally *in vitro*, were increased by one standard deviation resulting from experimental uncertainties of microsomal system - HDSC (204 S.D.), MPL (5.04 S.D.), $Vmax_M$ (143 S.D.), and Km_M (84.8 S.D.). The default input parameters are listed in the footnote to Table 3.2-1.**

The same input parameters, listed in the footnote to Table 3.2-1, were sampled at random in the Monte Carlo analysis of impact on these two variables (CV and AM, Figure 3.2-3 and 3.2-4, respectively) under the single exposure scenario. The Monte Carlo simulations were repeated until the subsequent runs stopped changing significantly both the lower and the upper bound of the output. Graphic results of Monte Carlo simulation of impact of the experimental error and the interidividual variability are compared in Figures 3.2-1A, 3.2-1B and 3.2-4A and 3.2-4B.

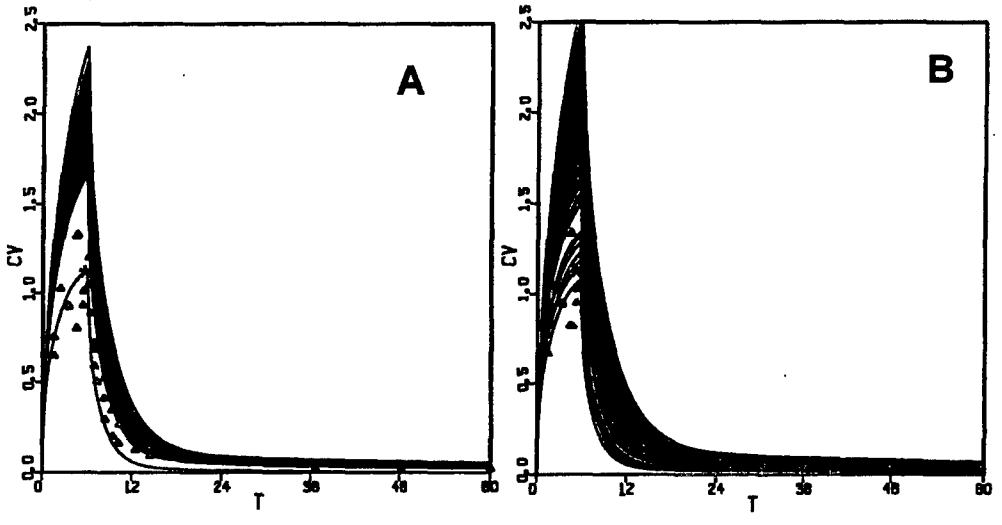

Figure 3.2-3. **Monte Carlo simulation of impact of experimental error and interidividual variability from measurement of metabolism in human microsomes, on TCE venous blood concentration (CV [mg/L]), simulated by PBPK model. Curve marked with asterisk - simulation optimized by Allen and Fisher (1993); Small triangles - experimental data from Muller et al., (1974 and 1975). T - time [h].**

About one hundred simulations were necessary to achieve the stable output. Interestingly, the output of a derivative variable (CV), calculated by the PBPK model as a rate, was less affected by the error propagation than the integrated variable (AM), which seems to cumulate errors over time (compare Figure 3.2-3 and 3.2-4). Given the tremendous range of the inter-individual variability of metabolism parameters in microsomes from different human donors (Lipscomb et al., 1997), the level of uncertainty introduced by experimental error from our *in vitro* system seems to be low.

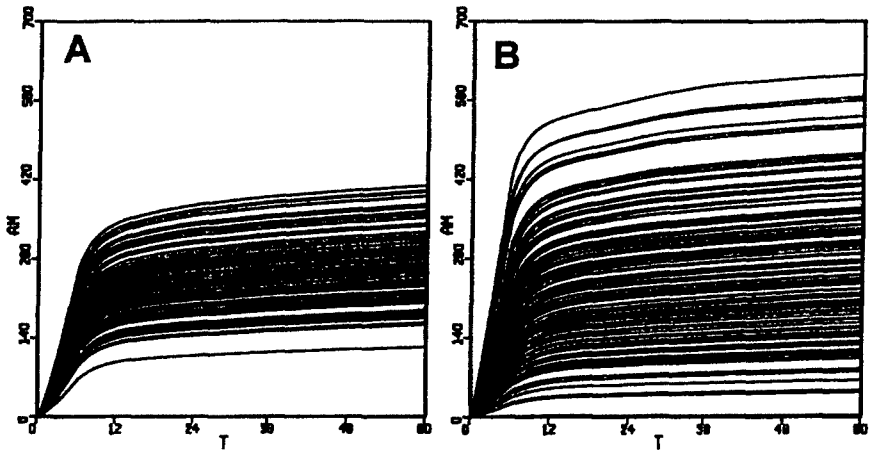

Figure 3.2-4. Monte Carlo simulation of impact ofexperimental error and interidividual variability from measurement of metabolism in human microsomes, on the amount of TCE metabolized (AM [mg]), simulated by PBPK model. T - time [h].

APPENDIX

```
'^^^^^^^^^^^^^^^ IN VITRO DROP-IN MODULE ^^^^^^^^^^^^^^^^^^^^^^^  '
          'IN VITRO PARAMETERS'
INITIAL
CONSTANT  HDSC= 5000.     $'Headspace TCE concentration [ppm]   '
CONSTANT  PCMA= 14.1      $'Ratio of micros/air concentrations  '
                  '[uM in micros/ppm in headspace].'
CONSTANT  NHEP= 116.      $'# of hepatocytes in liver  [#1e6/g liv.] '
CONSTANT  MPL = 20.8      $'Microsomal protein [mg mic./g liv.] '
MPH = MPL/NHEP $'Microsomal protein in hepatocytes [mg mic./1.e6 cells]'
CONSTANT VMAXM= 1589.     $'Vmax in microsomes [pmol/min/mg mic]'
CONSTANT  KmM = 399.      $'Km as TCE concentration in headspace [ppm]'
'^^^^^^^^^^^^^^^^^^^END OF IN VITRO INITIAL^^^^^^^^^^^^^^^^^^^^^^^^'
'^^^^^^^^^^^^^^^^    RECALCULATION MODULE   ^^^^^^^^^^^^^^^^^^^^^^^^  '
'Beginning of execution section of in vitro program                '
'  RMET  = Rate of metabolism [nmol/h/1.e6 cells]:                 '
   RMETM = (HDSC * VMAXM * 1.e-3 * 60. * MPH)/(KmM + HDSC)
'Metabolic rate in microsomes recalculated per million hepatocytes'
'  RMETD = Rate of metabolism in human donor [mg/h/kg BW]:         '
   RMETG = RMETM * NHEP             $'per g of liver [nmol/h/g] '
   RMETL = RMETM * NHEP * VL * 1.e3 $'per whole liver [nmol/h]  '
   RMETD =RMETM* 1.e-6 * MWTCE * NHEP * VLC * 1.e3  $'per kg BW '
'           Rate from microsomes recalculated as [mg/h/kg BW]'
'^^^^^^^^^^^^^^^^^^^^END OF RECALCULATION MODULE^^^^^^^^^^^^^^^^^^^^'
 '----------------------LINK WITH PBPK MODULE-------------------'
'   At saturation conditions:                                      '
  VMAXC = RMETD  $'At saturation rate equals VmaxC [mg/h/kg BW]'
   VMAX = VMAXC*BW**0.74            $'[mg/h/ human donor]     '
   KM = KmM * PCMA/PL * MWTCE/24450  $'Recalculates value from '
'headspace to microsomes to liver and to equillibrated blood [mg/L]'
'---------------------------END OF LINK-----------------------'
' ---Insertion Point in the DERIVATIVE Section of PBPK Module---'
    'AM = Amount metabolized [mg]'
    RAM = (VMAX*CVL)/(KM+CVL) + KF*CVL*VL
     AM = INTEG(RAM, 0.)   $'Amount metabolised per donor'
     amb= am/bw            $'Amount metabolised [mg/kg BW]'
     AML= AM/VL            $'Amount metabolised [mg/kg liver]'
    AMM = AMB/MWTCE        $'MMOLES METABOLISED [mmol/kg BW]'
   '----------------Continue PBPK Module----------------'
'^^^^^^^^^^^^^^^^^^^ END OF DROP-IN MODULE ^^^^^^^^^^^^^^^^^^^^^^  '
```

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SECTION 4

HALON REPLACEMENT TOXICITY PROJECT

4.1 SIMULATED BLOOD LEVELS OF CF_3I IN PERSONNEL EXPOSED DURING ITS RELEASE FROM AN F-15 JET ENGINE NACELLE AND DURING INTENTIONAL INHALATION

A. Vinegar, G.W. Jepson, S.J. Hammann[1], G. Harper[1], D.S. Dierdorf[2], and J.H. Overton[3]

ABSTRACT

Of the agents under consideration for protecting unoccupied areas, CF_3I has physicochemical properties that give it potential as a "drop-in" replacement for Halon 1301. One of the issues concerning the use of CF_3I is the potential hazard to ground crews should an inadvertent discharge occur while working in or near an engine nacelle. A discharge test of CF_3I was conducted on an F-15A jet at Robins AFB to record CF_3I concentration time histories at locations near the aircraft. The conditions of the discharges simulated an inadvertent ground discharge with the engine nacelle doors open and also with the doors closed. The use of three types of gas analysis instrumentation allowed gas sampling from several locations during the discharge tests. Concentrations measured at selected sensor locations were used as the input to a physiologically based pharmacokinetic (PBPK) model to simulate blood levels that would be attained by individuals inhaling CF_3I at sensor locations. Blood levels reached during these exposures were compared to the blood level associated with the Lowest Observable Adverse Effect Level (LOAEL) for cardiac sensitization in order to evaluate the possibility of safe egress. The highest blood concentrations simulated were twice the target blood concentration associated with cardiac sensitization. However, simulated blood concentrations of subjects who actually inhaled CF_3I reached levels that were 85 times the target level.

INTRODUCTION

International concern for the apparent depletion of stratospheric ozone has led to agreement to eliminate ozone depleting chemicals (ODCs). Recent studies have shown Halons to have the highest ozone depleting potential per unit mass, making them the first chemical family chosen for elimination. Originally planned to be phased out of production by the year 2000, the signatories to the Montreal Protocol accelerated the production ban of Halon to January 1994. This now leaves only existing stocks of Halon for use in essential applications such as aircraft fire and explosion suppression systems. Continually evolving political pressure keeps these stocks at some risk for elimination, accelerating the need to make Halon alternatives available to aircraft users in a timely fashion.

Alternatives to Halon include a broad assortment of halogenated hydrocarbons which are regulated by the U.S. EPA (1994). The acute toxic endpoint of concern is cardiac sensitization. Dogs are monitored and given an

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epinephrine challenge after five minutes of exposure to see if cardiac arrhythmias are induced. The test is repeated at several chemical concentrations to determine a No Observable Adverse Effect Level (NOAEL) and a Lowest Observable Adverse Effect Level (LOAEL). Since these tests are performed in a very sensitive subject (epinephrine-challenged dog), the LOAEL and NOAEL values are used directly for establishing human exposure limits (U.S. EPA, 1994). The exposure levels can be associated with blood concentrations by use of a physiologically based pharmacokinetic model. The arterial blood concentration, during exposure to the LOAEL exposure concentration, at the time of the epinephrine challenge (5 minutes) would represent a target concentration that one would not want to exceed (Vinegar et al., [in press]; Vinegar and Jepson, 1996).

Since CF_3I is the most similar replacement to Halon 1301 in terms of the volume and mass required to suppress a fire, it represents from a physicochemical perspective one of the most attractive Halon substitutes for unoccupied aerospace applications. Cardiac sensitization test results on CF_3I indicate that concentrations above 0.4% may pose an increased probability of adverse cardiovascular effects, thus making CF_3I a suitable candidate for use only in normally unoccupied areas. Although engine nacelles clearly are unoccupied, an inadvertent discharge of the nacelle extinguisher cylinder during ground maintenance of the aircraft could conceivably expose personnel to CF_3I. The risk of cardiac sensitization is dependent upon agent concentration and duration of exposure. To date, environmental concentration and time profiles for this agent have not been determined from accidental discharge scenarios.

This report describes testing performed to determine the CF_3I gas concentration levels around an F-15 after a ground level discharge in an engine nacelle. The discharge occurred through the existing F-15 Halon 1301 fire suppression system utilizing bottles charged with CF_3I instead of Halon 1301. The objective of this test was to characterize the actual CF_3I plume resulting from a simulation of an inadvertent discharge during ground maintenance. The resulting exposure data were then used in a physiologically based pharmacokinetic (PBPK) model to evaluate whether human blood concentrations resulting from the exposure would exceed those associated with cardiac sensitization.

METHODS

Exposure Scenario

The F-15A modified for use in this test is normally used for painting training at Robins AFB. All testing occurred inside a maintenance hangar 78 ft. deep, 60 ft. wide, and 30 ft. high. The primary modification to the aircraft was the installation of a standard F-15 fire suppression Halon bottle charged with approximately 9 pounds of CF_3I pressurized to 600 psig (pounds per square inch gauge) with nitrogen. Normally, the F-15 bottle contains 6.6 pounds of Halon 1301 pressurized to 600 psig with nitrogen. Since the test would only discharge agent into the

left hand engine nacelle, the test setup included only one pyrotechnic squib mounted to the appropriate discharge port. A remote controller fired the bottle's pyrotechnic cartridge to initiate a discharge. Four bottles of CF_3I were discharged during the test.

The test aircraft did not contain an engine. To provide a more realistic simuation, three large barrels were installed in the test nacelle with the supports between the barrels and bulkhead crossbeams simulating the engine mounting hardware. The engine interface at the inlet duct and the nacelle outlet was blocked to prevent the free flow of agent out these openings. The allowed remaining small flow area accounted for the ventilation gaps between the engine and nacelle.

The four tests performed addressed the potential of ground crew exposure to a plume of CF_3I gas inadvertently released in one of the engine nacelles during maintenance operations or during pre-takeoff inspection. The tests considered three general crew locations appropriate for maintenance activities close to the discharge nozzle. These locations were: 1) kneeling or standing near the engine bay, 2) working in or under the engine bay, and 3) prone near the engine bay. The first three discharges occurred with the engine bay doors open to simulate an accidental discharge during normal maintenance operations. The final discharge occurred with the engine nacelle doors closed to represent an accidental discharge during a pre-takeoff inspection.

Analytical Instrumentation

Four gas analyzers measured and recorded the CF_3I plume concentration and boundaries. The gas analyzers all sampled air through ¼-in diameter Tygon tubing located around the aircraft at elevations of ½ ft., 3 ft., and 5 ft. Spatial location of the sensors is shown in Figure 4.1-1. Distances from the point of CF_3I release (star) are shown in feet.

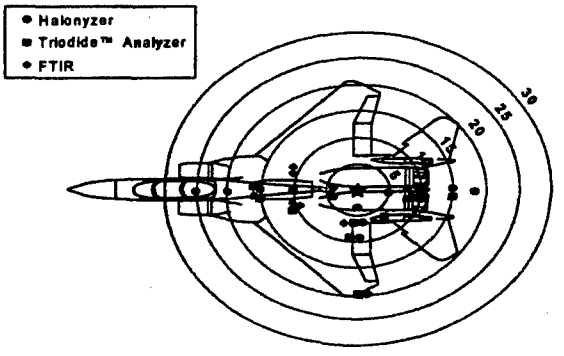

Figure 4.1-1. Top view of F-15 showing placement of sensors with respect to the point of release (star). Distances from point of release are indicated in feet.

Three types of gas analyzers were used during this test. The Halonyzer (Pacific Scientific) continuously samples gas from 12 locations producing accurate data for concentrations above 10,000 ppm (0.1 Hz sample rate, ± 0.2 Vol % theoretical accuracy). The Halonyzer draws gas samples through small bore tubing via a vacuum pump to a gas analysis sensor. The sensor contains a set of laminar flow capillary tubes that create a pressure drop that is proportional to the gas viscosity. Any difference in the pressure drop of the sampled gas from that of pure air is proportional to the relative concentration of the agent. To ensure uniformity of measurement, the sensor has internal heaters and heat exchangers as well as an inlet filter and sonic flow orifice.

The Triodide™ Analyzer (Pacific Scientific) sequentially samples from 6 locations producing accurate data for concentrations lower than 10,000 ppm (10 second sample cycle, ± 0.0125 Vol %). The Triodide™ Analyzer also draws gas samples through small bore tubing via a vacuum pump. Solenoid valves port the gas flow from one of the six locations to the infrared (IR) absorption gas chamber. The difference in IR adsorption between the sampled gas and pure air is proportional to the relative concentration of the agent. The sample chamber volume and sampled gas flow rate together determine the chamber purge time. The chamber purge time ultimately determines the minimum sample rate and sensor accuracy.

The two Fourier Transform Infrared spectroscopy (FTIR) analyzers (Bio-Rad) continuously sample gas from a single location producing accurate data at low concentrations (sample rate 0.1 Hz to 3 Hz, Resolution: 4 cm^{-1}). The FTIRs also draw their gas samples through small bore tubes via a vacuum pump to their sample chamber.

Physiologically Based Pharmacokinetic (PBPK) Modeling

Concentrations measured at selected sensors were used as the exposure input to a PBPK model to simulate blood levels that would be attained by an individual inhaling CF_3I at the locations of the sensors. Then, assuming that if the chemical was being released an individual would attempt to leave the site of chemical release, paths of egress were selected. Concentration-time data for the paths of egress were also used in the model to simulate the blood levels reached by individuals undergoing egress from the site of release. The model used for these simulations is a breath-by-breath model developed for simulating short term (0 to 5 min) human exposures (Vinegar et al., [in press]). Blood levels reached during these exposures were compared to the blood level that is associated with the LOAEL for cardiac sensitization with CF_3I. Using this modeling approach, the possibility of safe egress from these exposures was evaluated (Vinegar and Jepson, 1996).

Assuming that personnel were in the vicinity of the aircraft during an accidental release of CF_3I, several paths of egress were assumed. Simulations of blood concentrations were performed taking into consideration the exposure concentrations over time for the paths of egress. The particular scenarios considered were walking out the

left- hand side of the aircraft at 4 ft/sec, walking out toward the tail at 4 ft/sec, falling under the nacelle and crawling out at 2.5 ft/sec, and crouched egress from left hand main gear at 4 ft/sec. A three-second lag before egress began was assumed except for the case of falling, where a five-second lag was assumed.

Modeling Actual Human Exposure

In addition to simulating the blood levels of CF_3I that would potentially result from individuals being in the vicinity of an F-15 on the ground during an accidental release, it was also desired to model the blood levels attained from individuals known to have actually inhaled CF_3I. Initially when CF_3I was being marketed based on its abilities as a fire fighting agent, little toxicity information was available. Specifically, the cardiac sensitization potential had not yet been determined. During that time two individuals (both male) demonstrated the fire fighting ability of CF_3I by inhaling pure chemical from a balloon and extinguishing a lit candle. At the time of inhaling the chemical one subject was 38 yrs old, 5 ft. 9 ½ in., 235 lbs. The other individual was 35 yrs old, 5 ft.4 in., 150 lbs. The first subject was able to repeat breathing maneuvers inhaling air from the same balloons that had formerly been used with CF_3I. Balloon volumes were determined by water displacement. Initial balloon volumes were 1.5 to 2.1 liters and the residual volumes in the balloons ranged from about 300 mL to 800 mL. Thus, the average volume of inhaled CF_3I was about 1.25 liters with a potential range of 0.7 to 1.8 liters. Inhalation occurred in about 3 seconds with a breath hold of about 5 seconds. The second subject reported 2 to 3 second inhalations, 5 to 10 sec breath holds, and 2 to 3 sec exhalations. The first subject originally performed these maneuvers with CF_3I nine times while the second subject performed them six to eight times.

RESULTS

The PBPK model was used to establish the arterial blood concentration associated with the CF_3I LOAEL (4000 ppm) for cardiac sensitization. The resulting exposure to CF_3I at 4000 ppm. for 5 min produced a simulated arterial blood concentration of about 19 mg/L (Figure 4.1-2). This level represents the target chemical concentration in blood above which cardiac sensitization could occur.

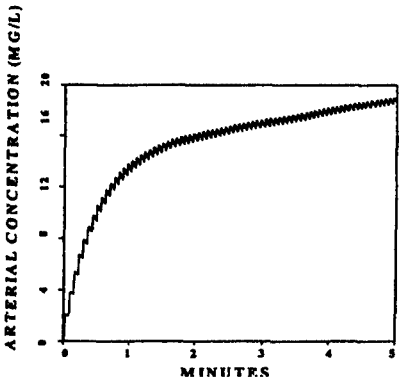

Figure 4.1-2. Simulated arterial blood concentration of CF_3I after 5-min inhalation of 4000 ppm. (LOAEL for cardiac sensitization).

Exposure concentrations measured at selected probe locations with corresponding simulated blood concentrations are shown in Figure 4.1-3. Concentrations at head level inside the open nacelle (Figure 4.1-3K) were expectedly the highest observed (over 70,000 ppm), simulated arterial blood concentration peaked at nearly 40 mg/L (Figure 4.1-3L), which is essentially twice the LOAEL blood concentration of 19 mg/L. The lowest peak blood concentration was seen for the waist level exposure behind the aircraft (Figure 4.1-3E) where the arterial concentration reached less than 6 mg/L (Figure 4.1-3F). The peak exposure concentration was nearly 16,000 ppm, but dropped rapidly to less than 1000 ppm with a brief excursion to about 2500 ppm. In contrast, a peak exposure of only 9000 ppm was seen at head level under the left wing (Figure 4.1-3I) with a drop to around 4000 ppm around which the concentration fluctuated for several minutes. The blood concentration (Figure 4.1-3J) peaked at over 16 mg/L, dropped slightly and then hovered at over 16 for the entire measurement period. Other locations showed concentrations that were in between the above extremes.

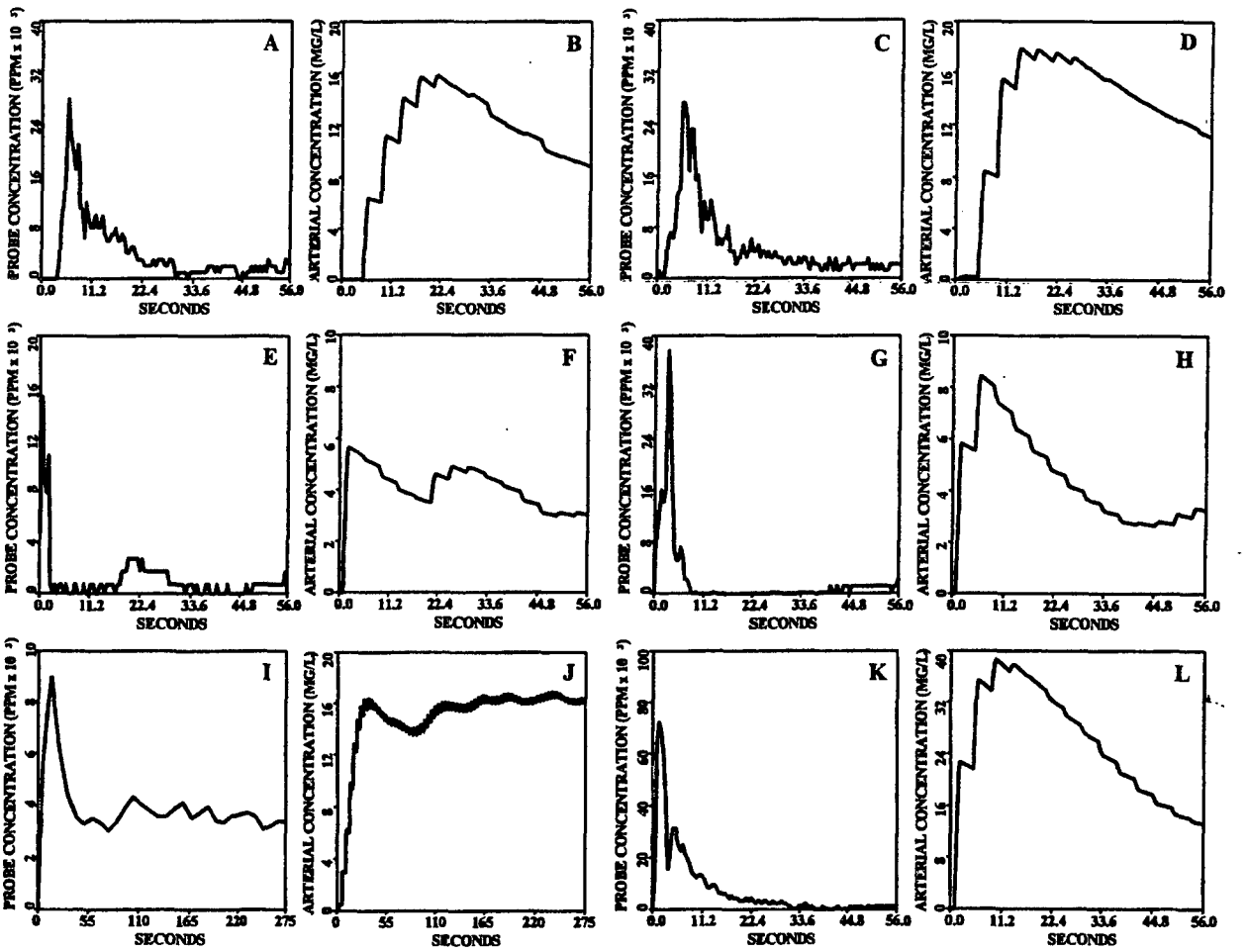

Figure 4.1-3. Measured concentration of CF_3I at various probe locations during release from F-15 jet engine nacelle with corresponding simulated arterial blood concentrations if individuals inhaled the chemical at each location. A-B: ground level under left wing; C-D: ground level under nacelle; E-F: waist level behind aircraft; G-H: waist level under nacelle; I-J: head level under left wing; and K-L: head level under nacelle.

Of the 4 egress scenarios modeled, either walking out the left hand side of the aircraft (Figure 4.1-4A) or walking out towards the tail (Figure 4.1-4E) would result in blood concentrations exceeding 19 mg/L (about 23 [Figure 4.1-4B] and 33 mg/L [Figure 4.1-4F] for the two scenarios, respectively). If someone fell under the nacelle and crawled away (Figure 4.1-4C), the peak arterial blood concentration would be about 15 mg/L (Figure 4.1-4D), and if someone walked in a crouched position from the left hand main gear (Figure 4.1-4G), blood concentration would reach a peak of about 3 mg/L (Figure 4.1-4H).

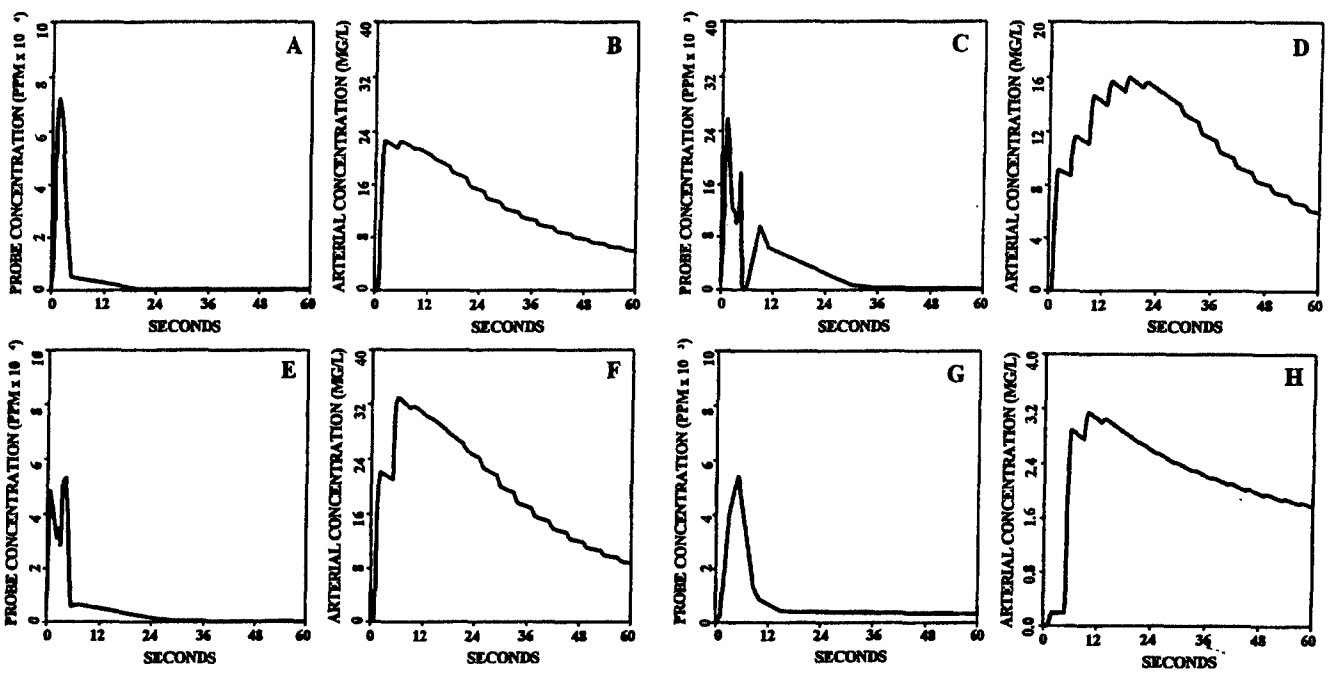

Figure 4.1-4. Measured concentration of CF_3I at different points along different paths of egress from the point of release with corresponding simulated arterial blood concentrations of individuals moving along the paths. A-B: walk out left hand side; C-D: fall under nacelle and crawl; E-F: walk out aircraft tail; G-H: crouched egress from left hand gear.

In comparison, the blood concentrations of individuals who actually inhaled CF_3I during a demonstration were much higher. Using the average conditions for one individual, the simulated blood concentration reached a peak of about 1500 mg/L (Figure 4.1-5) and after 5 min was still about three times the critical concentration of 19 mg/L.

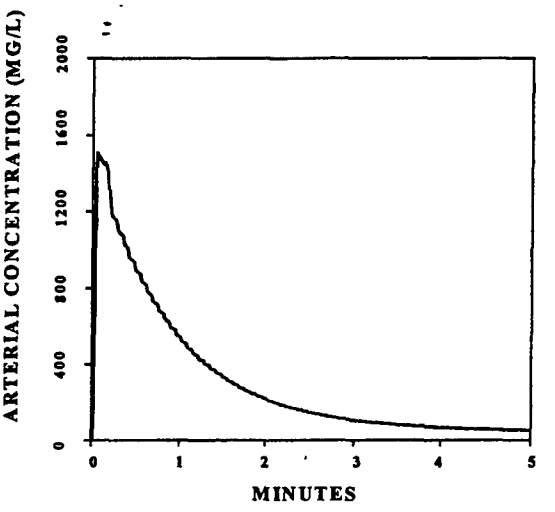

Figure 4.1-5. Simulated blood concentration of an individual taking a 3-sec breath of pure CF_3I from a balloon with a breath hold of 5 sec, an exhalation of 3 sec, and a return to normal breathing.

DISCUSSION

The test methodology produced repeatable and consistent measurements of the CF_3I gas plume resulting from the discharge of the gas into the engine nacelle. The use of three different types of gas analyzers provided accuracy across the measured concentration range. The sensors with higher accuracy not only provided good data at low concentration, but also acted as a check against the concentration data from the lower accuracy Halonyzer.

Of all probe locations, the one showing the highest simulated arterial concentration was located inside the open nacelle at head height. The peak concentration at that point was nearly 40 mg/L. Concentration profiles at any point varied over time. Simulations of potential egress paths for personnel in the vicinity of the open nacelle at the time of accidental release of CF_3I indicated that some paths would result in peak blood concentrations exceeding the LOAEL target blood concentration of 19 mg/L and others would fall below the target.

The simulated blood levels of the two individuals who intentionally inhaled CF_3I, without ill effect, were clearly well above those reached by individuals who would have been at any position during the release of CF_3I during the F-15 tests. In fact, the simulation for the one individual produced a peak concentration some eighty-five times that of the 19 mg/L target. The two individuals inhaled deep breaths of CF_3I 15 to 17 times between them without showing any adverse effect. Even a ten-fold reduction (to allow for sensitive individuals) in the peak concentration breathed by these individuals would be well above 19 mg/L. This illustrates that using the LOAEL value for cardiac sensitization, as determined in epinephrine-challenged dogs, is a conservative approach for protecting individuals exposed to fire fighting agents.

One might consider setting the margin of safety for egress from a potential CF_3I exposure by modeling a particular exposure scenario using the simulated blood concentrations from the exposed individuals as a target. Using this approach, the levels of CF_3I encountered during its release from an F-15 nacelle would not be considered a cardiac sensitization threat to maintenance personnel who might be in the vicinity of the release. Where human exposure data are not available, which is normally the case, then the cardiac sensitization LOAEL would still be used as the target exposure for determining the critical blood concentration. Even though this may be a conservative approach, it is still more liberal than the current guidelines for flooding agents which suggest designing to the NOAEL, or up to the LOAEL with 30 sec to 1 min egress, or above the LOAEL with egress of less than 30 sec. Most potential replacement agents, having much higher LOAELs for cardiac sensitization than does CF_3I, would benefit in extending potential egress time out to several minutes.

ACKNOWLEDGMENTS

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4.2 ACUTE AND SUBCHRONIC TOXICITY EVALUATIONS OF THE HALON REPLACEMENT CANDIDATE PHOSPHORUS TRIBROMIDE - A FINAL REPORT

R.E. Wolfe, M.L. Feldmann, D.H. Ellis, H.F. Leahy, C.D. Flemming, D.E. Dodd, and J.S. Eggers[1]

ABSTRACT

Phosphorus tribromide (PBr_3) is being considered by the DOD as a possible replacement for Halon 1301. This study was designed to determine the effects following single, high-concentration exposures which could occur in accidents, as well as repeated, low-concentration exposures which could occur on flight lines or where maintenance commonly occurs. Application of 10 or 50 µL neat PBr_3 to intact skin of an anesthetized New Zealand white (NZW) rabbit caused edema and necrosis of the treated skin within 10 minutes of dosing. Microscopic examination confirmed necrosis of the skin and underlying areas, including the skeletal muscle of the subcutis. Acute 4-h nose-only exposure of Fischer 344 (F-344) rats to PBr_3 vapor resulted in mortality at 4.1 mL. At 1.5 mL, labored breathing, body-weight loss, ulceration of anterior nares, and rhinitis of the nasal passage were observed. Toxicity was not observed in rats exposed for 4 h to 0.4 mL. Male rats (5 per group) were exposed to PBr_3 vapor, 4 h/day for 5 days, at 0, 0.06, 0.16, and 0.51 mL PBr_3. There were no signs of toxic stress. Rats of the 0.51 mL group had decreased body weights; gross lesions (reddened nares) and microscopic lesions (inflammation of mucosa and ulceration of epithelium of the nares) were also observed. Ten male and 10 female rats per group were exposed to PBr_3 vapor, 4 h/day, 5 days/week, for 4 weeks at 0, 0.03, 0.1, and 0.3 mL. There were no signs of toxic stress, alterations in body weights, or changes in organ weights in PBr_3-exposed animals. Minor serum chemistry and hematology effects were observed in the treated animals. Microscopic tissue findings were limited to rats of the 0.3 mL group and consisted of mild inflammation of the nasal passages. A concentration of 0.1 mL is the No Observable Adverse Effect Level (NOAEL) in the 28-day inhalation study.

INTRODUCTION

The Department of Defense requires the development of a complete toxicity profile for replacement candidates of halons, which includes the results from acute and subchronic toxicity testing. Many of these compounds, including phosphorus tribromide, have not been thoroughly investigated to determine their toxicological properties. The purpose of this study was to conduct acute and subchronic toxicity testing to provide acute toxicological hazard information to complete a health hazard assessment for phosphorus tribromide. The health hazard assessment will help determine if phosphorus tribromide (PBr_3) can be used as a Halon replacement compound. U.S. Air Force tests in a 500,000 BTU/h test burner demonstrated that PBr_3 is an effective fire extinguishant. The test burner was quenched by only 0.2 mL PBr_3. This volume is several thousand times less

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than the amounts required of other halon replacements to suppress fires. The new fire extinguishant systems based on PBr_3 usage will occupy less volume, weigh less, and require less mechanics than current systems used on U.S. Air Force aircraft and electronic equipment fires. Also, PBr_3 has no ozone depleting potential since it is rapidly hydrolyzed in the troposphere.

Phosphorus tribromide reacts with moisture in the air to produce phosphonic acid and hydrogen bromide gas (HBr). There is limited toxicity information available in the literature on phosphorus tribromide and hydrogen bromide. No acute toxicity information is available for phosphonic acid or hydrobromic acid; the aqueous form of HBr. The combined interaction of these compounds to cause potential health hazards is unknown.

An accidental human exposure to a mixture of PBr_3 and HBr was reported by Kraut and Lilis, 1988. While mixing PBr_3 and HBr, a female laboratory assistant was exposed to these compounds via splashing on the face, chest, and hair, and by inhalation of resulting vapors. She remained in the area of the exposure for 5-to-10 minutes before receiving treatment for the chemical burn. Immediate effects noted were dry cough, light-headedness, and slight congestion of the throat. Over the next two weeks, the subject experienced increasing shortness of breath. Chest x-rays revealed bilateral lobe infiltrates, and chemical pneumonitis was diagnosed. She was allowed to return to work a few months later, though dyspnea on-exertion persisted, and chest x-ray findings had not yet been completely resolved. No exposure assessment was conducted, and attempts to get exposure information were not successful.

Tissue injury of the respiratory tract was observed in a study of rats exposed to 1300 ppm. HBr for 30 min that compared nose-breathing effects to pseudo-mouth-breathing effects (Stavert et al., 1991). Tissue damage following nose-breathing exposure to HBr was confined to the nasal region. Observations included epithelial and submucosal necrosis. Pseudo-mouth-breathing exposure to HBr caused higher mortality rates, and major tissue disruption was noted in the trachea. Observations included epithelial, submucosal, glandular, and cartilage necrosis.

Results of genotoxicity testing of bacterial Salmonella strains (ManTech, 1996) indicated PBr_3 is not a mutagen for both frame shift and base-pair substitution tester strains in both buffered and unbuffered solutions. Acute aquatic toxicity tests on the fathead minnow and *Daphnia magna* have also been conducted with PBr_3. The 96-h LC_{50} value for fathead minnow was 71 mL (50 - 100 mL confidence limit); the no-observed effect concentration was 25 mL (Aqua Survey, 1996a). The 48-h EC_{50} value for *Daphnia magna* was 22.6 mL (18.2 - 27.9 mL confidence limit); the no-observed effect concentration was 6.25 mL (Aqua Survey, 1996b).

The objectives of this study were to determine the acute and subchronic toxicity of PBr_3. The information gathered from these experiments should provide a preliminary database for developing a health hazard assessment for PBr_3.

MATERIALS AND METHODS

Details of materials and methods are available in the technical report by Wolfe et al. (1997). Phosphorus tribromide (PBr_3; CAS No. 7789-60-8) was supplied by Aldrich Chemical Company (Milwaukee, WI). Male and female F-344 rats weighing between 100 and 125 g, and 75 and 100 g, respectively, were purchased from Charles River Breeding Labs, Raleigh, NC. Male NZW rabbits weighing between 2 and 3 kg were purchased from Myrtle's Rabbitry, Inc., Thompson's Station, TN.

Acute Toxicity Tests

The approach for determining the acute toxicity of PBr_3 was to perform the following: Skin Irritation Screen using rabbits, and an Acute Inhalation (Limit Test) using rats. The information gathered in these tests will provide useful information necessary to establish limits for emergency conditions resulting from spills or leaks involving PBr_3.

Subchronic Inhalation Toxicity Tests

A one-week (5 exposures) pilot study was performed to determine target concentration levels for a subchronic 28-day study. Five male F-344 rats were exposed to air only or PBr_3 (0.5, 0.1, and 0.05 mL) for 4 h/day, 5 consecutive days. The concentrations of PBr_3 utilized in the 28-day inhalation study were 0.3, 0.1, 0.03, and 0.0 (air control) mL. Ten male and 10 female F-344 rats per group were exposed to PBr_3 or air only for 4 h/day, excluding weekends, for 28 days (a total of 20 exposures). Exposures were performed using Cannon nose-only chambers (Cannon et al., 1983).

RESULTS

Details of results are available in the technical report by Wolfe et al., 1997 (AL/OE-TR-1997-0123).

Skin Irritation Screen

Application of 50 µL or 10 µL neat PBr_3 to intact rabbit skin caused an immediate reaction, producing a white vapor. No immediate change in the color of the rabbit skin occurred, though the treated areas were hardened when compared to adjacent untreated skin. Ten minutes after dosing, the skin of both rabbits (where site of test substance application was either rinsed with water or unrinsed) had severe edema and necrosis for each volume (50 or 10 µL) of PBr_3 used. The affected skin was limited to the treated area only and was clearly demarcated from the adjacent untreated skin.

The animal tested dermally with no rinsing of test material had both skin and muscle lesions noted at necropsy. These tissues were collected and examined histologically. Findings included focally extensive necrosis of the entire thickness of the haired skin, epidermis, and dermis. Focally extensive and severe-hyaline degeneration and necrosis of the panniculus muscle were observed. Hyaline degeneration and necrosis were also observed in the skeletal muscle of the underlying subcutis. In the second rabbit tested dermally, but with the test material rinsed with water from the skin, only skin lesions were noted at necropsy. The skin lesion was collected and examined histologically. As in the first rabbit, histologic findings included extensive necrosis of the entire thickness of the haired skin, epidermis, and dermis. Multifocal, moderate-hyaline degeneration, and necrosis of the panniculus muscle were also noted. Injury did not spread to the underlying subcutis. No further testing for skin irritation was performed.

Acute Inhalation Toxicity

One of five female rats from the 4.1 mL PBr_3 nose-only-inhalation exposure died within 5 min of the conclusion of exposure (Table 4.2-1.).

TABLE 4.2-1. DATA SUMMARY FOR THE ACUTE 4-h INHALATION EXPOSURES OF PHOSPHORUS TRIBROMIDE

Target Conc. mL	Nominal Conc. mL	Analyzed Conc. mL	Animal No.	Body Wt[a] Day 0	Body Wt Day 1	Body Wt Day 2	Body Wt Day 7	Body Wt Day 14
0.5	0.58	0.42	11	260.5	239.7	236.6	250.7	263.0
Males			12	252.9	234.2	229.9	243.1	258.1
			13	243.1	234.5	228.7	241.4	248.2
			14	232.7	225.9	223.3	236.0	224.0
			15	254.8	240.9	237.8	250.2	255.2
			Mean	248.8	235.0	231.3	244.3	249.7
			SD	11.0	5.9	6.0	6.2	15.3
1.0	1.07	0.89	06	256.6	247.8	241.4	251.6	260.9
Males			07	236.4	214.1	196.1	205.8	229.1
			08	232.6	215.2	200.1	210.1	236.3
			09	229.8	213.8	192.0	191.6	219.1
			10	248.1	236.0	225.7	242.1	254.0
			Mean	240.7	225.4	211.1	220.2	239.9
			SD	11.3	15.7	21.5	25.5	17.3
2.5	2.31	1.48	16	257.2	231.7	224.0	222.1	240.4
Males			17	255.8	229.8	214.0	198.2	234.6
			18	261.4	233.9	218.3	206.9	238.0
			19	267.0	240.4	224.3	197.8	219.4
			20	244.3	225.7	207.4	186.9	198.7
			Mean	257.1	232.3	217.6	202.4	226.2
			SD	8.4	5.4	7.1	13.1	17.4
5.0	4.97	4.09	21	168.0	156.2[b]			
Females			22	160.4[c]				
			23	161.5	150.8[b]			
			24	167.6	---[d]			
			25	166.7	157.5[b]			
			Mean	164.8	154.8			
			SD	3.6	3.6			

[a]Body weight in grams.
[b]Euthanized moribund postexposure Day 1.
[c]Animal died immediately postexposure.
[d]Animal found dead postexposure Day 1.

Another female rat was found dead, and the other three females were euthanized as moribund at one-day postexposure. Observations during exposure included labored breathing and mouth breathing. During the first hour of exposure, all animals tried to avoid breathing the exposure atmosphere by attempting to withdraw their nose and head from the nose cone of the exposure tubes. At necropsy, gross findings consisted of four-of-five animals with nares plugged with a black substance, and one-of-five animals had the epidermal-layer of nares missing. Histologic findings consisted of severe, diffuse, and peracute necrosis of the lamina propria epithelium of the nasal passages, and moderate-to-severe multifocal acute necrosis of the turbinate epithelium.

Animals in the 1.5 mL exposure group showed signs of labored breathing and some struggled to pull away from the PBr_3 atmosphere during exposure. Postexposure, the animals appeared mildly unsteady with labored breathing. Body weights were lower than expected for postexposure Days 1 through 7 (Table 4.2-1.). All animals survived the 14-day observation period. Gross findings consisted of tips of nares missing and deep ulceration of the external nares with exposure of underlying cartilage. Histologic findings consisted of mild-to-moderate, suppurative-to-subacute, mulitfocal rhinitis of the lamina propria of the nasal passages in all animals. One-of-five animals also displayed squamous-cell metaplasia of the nasal passages (most anterior regions).

Observations of animals exposed to 0.9 mL of PBr_3 during the first hour included rapid breathing, pulling away from the PBr_3 air source to avoid the exposure atmosphere, twisting in the tubes and contracted abdominal area. Animals appeared less active after the first hour of exposure and displayed rapid shallow breathing. All animals survived the 14-day observation period, and there were no gross findings at necropsy.

Animals exposed to 0.4 mL PBr_3 appeared normal, and all survived the 14-day observation period. There were no gross findings at necropsy. Pathology findings were normal.

Five-Day Inhalation Range-Finding Study

Mean $\pm$ standard deviation exposure concentrations of PBr_3 were 0.06 $\pm$ 0.01, 0.16 $\pm$ 0.03, and 0.51 $\pm$ 0.08 mL for target exposure concentrations of 0.05, 0.1, and 0.5 mL respectively. Analytical to nominal concentration ratios for these exposures were 0.4, 0.6, and 0.4, respectively. There were no deaths or signs of toxic stress during the 5-day exposure period. Mean body weights decreased in all exposure groups and were significantly different in the high concentration group when compared to control on Study Days 3 and 4 (Table 4.2-2.).

TABLE 4.2-2. MEAN BODY WEIGHTS[a] OF MALE F-344 RATS DURING FIVE-DAY, NOSE-ONLY INHALATION EXPOSURE TO PHOSPHORUS TRIBROMIDE OR AIR

Day of Study	CONTROL	0.05 mL	0.1 mL	0.5 mL
0	258.2 ± 8.0	259.0 ± 10.4	255.6 ± 12.5	258.8 ± 10.7
1	250.4 ± 7.9	251.0 ± 10.5	245.3 ± 13.8	247.7 ± 12.6
2	245.4 ± 8.3	245.9 ± 9.4	241.6 ± 12.9	239.9 ± 10.2
3	242.7 ± 9.0	242.9 ± 9.8	237.3 ± 13.1	234.7 ± 9.9[b]
4	241.2 ± 9.0	240.5 ± 10.5	236.3 ± 12.0	229.0 ± 8.5[b]
5	225.7 ± 8.6	225.2 ± 8.6	220.6 ± 10.6	214.4 ± 7.9

[a]Mean ± SD, N=5.
[b]Significantly different from control at $p < 0.01$.

Statistically significant differences between control and PBr_3-exposed animals were observed in several mean values of serum chemistry and hematologic parameters (Wolfe et al., 1997). Increases in serum chloride values were attributable to interference with the presence of serum bromide (See Discussion section). Increased values in calcium and potassium were noted in the 0.51 mL group. Decreased values when compared to control were observed in alkaline phosphatase and creatine kinase in the high-dose group; ALT values in the 0.16 mL and 0.51 mL groups. Additional statistically significant differences in mean values of serum chemistry or hematologic parameters were sporadic and were not considered biologically important.

Gross observations at necropsy revealed irregular shaped and reddened nares in 3 of 5 animals in the 0.51 mL group. There were no gross lesions observed in the other exposure groups, including the control group. Absolute organ weight means and mean organ weight to body weight ratios (Wolfe et al., 1997) revealed no statistically significant differences compared to control animals. Microscopic lesions were observed in the 0.51 mL group and in the anterior-most segment of the nasal passages. In this group, suppurative (acute) inflammation of the mucosa of the nasal passages occurred at a statistically higher incidence than control animals ($p < 0.05$). Chronic ulceration of the epithelium of the external nares was observed in 3 of 5 animals. Minimal squamous metaplasia of the respiratory epithelium was observed in the trachea of one rat in the high concentration group. In the 0.16 mL PBr_3 group, 1 of 5 rats had slight inflammation of the nasal mucosa (most anterior regions). There were no microscopic lesions in rats of the 0.06 mL and control groups.

Subchronic Inhalation Toxicity Study (28-day)

Mean ± standard deviation concentrations of PBr_3 were 0.30 ± 0.03, 0.11 ± 0.01, and 0.034 ± 0.004 mL for target exposure concentrations of 0.3, 0.1, and 0.03 mL, respectively. Analytical to nominal concentration ratios for these exposures were 0.51, 0.50, and 0.62, respectively. The daily mean temperature for the exposure

atmospheres ranged from 73.1 °F to 76.5 °F, and the daily mean relative humidity ranged from 2.4 to 3.6 %. No deaths occurred during the study. In addition, there were no treatment-related clinical signs observed in the study. Mean body weights for male and female rats in the control and PBr_3 exposure groups were similar (Tables 4.2-3 and 4.2-4.).

TABLE 4.2-3. BODY WEIGHTS[a] OF MALE RATS EXPOSED FOR 28 DAYS TO PHOSPHORUS TRIBROMIDE VIA NOSE-ONLY INHALATION

Day of Study	Control	0.03 mL	0.1 mL	0.3 mL
-1	206 ± 7.9	206 ± 7.8	206 ± 8.7	205 ± 7.8
6	215 ± 8.4	215 ± 10.4	215 ± 8.6	215 ± 11.4
13	223 ± 7.2	224 ± 9.3	223 ± 9.8	220 ± 7.2
20	233 ± 7.1	232 ± 11.5	229 ± 9.3	227 ± 7.8
27	244 ± 8.8	244 ± 13.2	239 ± 10.5	236 ± 8.2

[a]Mean ± SD, N = 10.

TABLE 4.2-4. BODY WEIGHTS[a] OF FEMALE RATS EXPOSED FOR 28 DAYS TO PHOSPHORUS TRIBROMIDE VIA NOSE-ONLY INHALATION

Day of Study	Control	0.03 mL	0.1 mL	0.3 mL
-1	137 ± 5.2	136 ± 5.8	136 ± 4.0	136 ± 5.4
6	147 ± 5.9	148 ± 5.5	147 ± 7.0	148 ± 8.8
13	147 ± 6.5	150 ± 5.5	149 ± 4.0	149 ± 8.5
20	150 ± 7.2	153 ± 6.2	150 ± 4.7	149 ± 8.2
27	154 ± 7.8	157 ± 6.6	154 ± 4.0	152 ± 9.7

[a]Mean ± SD, N = 10.

Mean values of several serum chemistry and hematologic parameters were statistically significantly different in PBr_3 animals compared to those of the control group (Wolfe et al., 1997). Increases in serum chloride concentrations in rats of the 0.3 and 0.1 mL groups were attributable to interference with the presence of serum bromide (See Discussion section). A small increase in chloride values and a small decrease in ALT levels were observed in rats exposed to 0.3 mL PBr_3. Though not concentration-related, an 11-26% decrease in triglycerides was observed in all groups of rats exposed to PBr_3. Slight increases in mean corpuscular volume, monocytes, and eosinophils were observed in the high concentration exposed rats. A concentration-related decrease in platelets was observed in rats of the 0.3, 0.1, and 0.03 mL groups. Additional statistically significant differences in mean values of serum chemistry or hematologic parameters were sporadic and were not considered biologically significant.

No treatment-related gross lesions or differences in absolute or relative organ weights (Wolfe et al., 1997) were observed at necropsy.

Tissues from rats of the 0.3 mL PBr_3 and air-only control groups were examined microscopically. The only treatment-related finding was the presence of exudate or evidence of mild inflammation of the anterior nasal passages in 3 of 10 male rats and 1 of 10 female rats. This finding was not statistically significant compared to the control group values.

DISCUSSION

Application of neat PBr_3 causes an immediate reaction that results in edema and necrosis of the skin. Microscopic examination of the treated skin indicates necrosis through the epidermis and dermis, the panniculus muscle, and into the skeletal muscle of the underlying subcutis. Rinsing with water 30 sec after application of neat PBr_3 did not alter the severity of the effect on the skin, though the extent of damage was not observed in the underlying subcutis. The appearance of these lesions were considered comparable with an acute chemical injury (burn).

To administer PBr_3 vapor to animals, special conditions were set up. Relative humidity was removed from the air entering the exposure chambers to limit as much as possible the reaction of PBr_3 with water vapor. For safety considerations, nose-only inhalation exposures were conducted. This effort limited the total amount of PBr_3 vapor that was required for the inhalation studies and confined the work area due to the small size of nose-only chambers compared to whole-body chambers. To monitor the concentration of PBr_3 vapor during animal exposures, an analytical method was developed that measured bromide ion following the reaction of PBr_3 vapor with water. Details of the analytical procedure are given in the report by Wolfe et al., 1997.

Acute 4-h nose-only exposure of Fischer 344 rats to PBr_3 vapor resulted in mortality at 4.1 mL. At 1.5 mL, labored breathing, body weight loss, ulceration of anterior nares, and histopathologic lesions (rhinitis) of the nasal

passage were observed. Signs of toxicity were also observed at 0.9 mL PBr_3. Clinical signs, gross lesions, and microscopic tissue lesions were absent in rats exposed for 4 h to 0.4 mL PBr_3. This series of acute animal exposures provides good concentration-response data for single, high PBr_3 concentration exposure scenarios.

In the 5-day concentration range-finding study, there were no overt signs of toxic stress. Rats of the 0.51 mL group had a statistically significant decrease in body weight means compared to the controls. Gross lesions (reddened nares) and microscopic lesions (suppurative inflammation of mucosa and ulceration of epithelium of the external nares) were observed in rats of the 0.51 mL group. Slight inflammation of the nasal mucosa (most anterior regions) was also observed in a rat of the 0.16 mL group. Rats in the 0.06 mL group were without adverse effects, and values of measured parameters were similar to those of the air-only control rats. These results were used to select target exposure concentrations for the 28-day inhalation study. The target concentrations were 0, 0.03, 0.1, and 0.3 mL PBr_3.

In the 28-day study, there were no signs of toxic stress, alterations in body weights, or changes in organ weights in PBr_3-exposed animals. Minor serum chemistry and hematology effects were observed in the treated animals. Microscopic tissue findings were limited to rats of the 0.3 mL group and consisted of mild inflammation of the anterior nasal passages.

Note that a decrease in mean body weight was observed in all exposure groups, including the control group, in both the 5-day and 28-day inhalation studies. Exposure systems requiring animal restraint may cause animal stress (review by Phalen et al., 1984), such as excessive heat, decreases in body weight, or alterations in pulmonary function. Note, also, the increase in serum chloride concentrations in PBr_3 exposed rats only in both the 5-day and 28-day studies. The increase in serum chloride levels in PBr_3 exposed rats only is believed to be an artifact, due to interference from ionized bromide in the test material with the serum chloride assay. Information collected from the procedure manual for the chloride assay (Vitros CAT No. 8120446, 11/96) indicates bromide and iodide (from therapeutic drugs) are known interfering substances.

On the basis of results observed in these studies, neat PBr_3 is corrosive when applied to the skin, the acute (4-h) lethal concentration of PBr_3 is approximately 4 mL, and a concentration of 0.1 mL is the NOAEL in the 28-day inhalation study.

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4.3 ACUTE TOXICITY EVALUATION OF REPLACEMENT CANDIDATES FOR OZONE-DEPLETING SUBSTANCES

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ABSTRACT

The DoD requires the development of toxicity profiles for chemical substitute candidates proposed to replace ozone-depleting substances such as chloro- and fluorocarbons and halons. Three test substances were identified as possible replacement candidates for ozone-depleting fire extinguishants: hexakis (2,2,2-trifluoroethoxy); cyclotriphosphazene, a 9:1 mixture of 1,1,1,3,3,3-hexafluoropropane; and 1-bromopropane, and a 93:7 mixture of perfluoro-2-butene and 1-bromopropane. These test substances are referred to as hexakis, HFP/BP, and PFB/BP, respectively, hereafter. An oral toxicity evaluation and genotoxicity testing were performed on hexakis. Acute inhalation toxicity tests were conducted on HFP/BP and PFB/BP. No deaths occurred in rats treated with hexakis at the limit test value of 5 g/kg. Also, no deaths were observed in rats exposed to HFP/BP or PFB/BP at the limit test value of 5 mg/L. Hexakis did not produce mutagenicity in the Ames test. For all three test substances, body weights of male and female rats were unaffected by treatment during the 14-day observation period. These test materials did not produce acute toxicity at limit test doses via the oral (hexakis) or inhalation (HFP/BP, PFB/BP) routes of administration.

INTRODUCTION

Fire extinguishant agents, refrigerants, and other solvents presently in the Department of Defense (DoD) and worldwide inventory contain halogenated fluorocarbons. Chloro- and bromofluorocarbons (halons) are substances thought to cause ozone depletion in the stratosphere. Environmental concern over this ozone depletion by activity of chlorine radicals from chlorofluorocarbons (CFCs) has led to an international treaty called the Montreal Protocol (1987) which calls for the phaseout of select CFCs and halons by the year 2000. The potential utility of a number of chemical substitutes that have little or no ozone depleting potential are being investigated to meet the demand for alternatives to CFCs and halons.

The DoD requires the development of a toxicity profile for the potential chemical replacements, which includes the results of acute toxicity testing. Because these replacements are currently being developed and are not manufactured commercially, very little, if any, toxicity information is available in the literature. To initiate responsible industrial hygiene practice within the production area and provide or recommend appropriate protective equipment in the workplace, it is necessary that the operations personnel are aware of the acute health hazards of this compound.

Hexakis, HFP/BP, and PFB/BP are chemical replacement candidates being considered for Ozone Depleting Substances (ODS). Hexakis is a solid material with a vapor pressure of 2 mmHg at 70 °C. An acute oral toxicity test was performed on hexakis, in place of the acute inhalation toxicity test that is more routinely performed on ODS replacement candidates. Hexakis is also amenable for genotoxicity testing and was tested for mutagenicity using the Ames *Salmonella*/microsome assay. HFP/BP and PFB/BP are nonflammable gas/vapor mixtures. Thus, acute inhalation toxicity tests were performed on them. No acute toxicity data are available on hexakis or HFP. Vinegar et al. (1996) conducted gas uptake kinetic experiments with HFP. PFB is considered mildly toxic by inhalation based on mutation data reports (Lewis, 1991). According to a Material Safety Data Sheet distributed by Aldrich Chemical Company, Inc., Milwaukee, WI (07/17/96), BP vapor is irritating to the eyes, mucus membranes, and upper respiratory tract. The 30-min LC_{50} value in rats is 253 mg/L. BP is a highly flammable colorless liquid (IOSHIC, 1989) and irritates the skin. Long-term exposure to BP can cause hepatic and renal damage (IOSHIC, 1989).

Data obtained from acute toxicity testing of these test substance candidates will provide a measure of toxic potency that can be compared with other chemicals, including other CFCs and halon replacement candidates. The species and sex of animals selected for this acute toxicity test were in conformance with the requirements of the U.S. Environmental Protection Agency (1982). Existing alternative methods to animal testing were inadequate for use in this study.

MATERIALS AND METHODS

Details of materials and methods are available in the technical reports by Wolfe et al. (1997a, 1997b) and Feldmann et al. 1997. Hexakis and HFP/BP were provided by the University of New Mexico, New Mexico Engineering Research Institute, Albuquerque, NM. PFB/BP was supplied by Mr. Barry Mitchell, WL/FIVCTF, Tyndall AFB, FL. Male and female Fischer-344 (F-344) rats, 7 weeks of age, were purchased from Charles River Breeding Labs, Wilmington, MA.

Oral Toxicity Limit Test

Five male and five female F-344 rats were fasted 12 h prior to the administration of an oral gavage dose (5 g/kg) of hexakis, suspended in 1% agar solution vehicle (1 mL/100 g body weight). Control animals (5/sex) received the vehicle only. Animals were observed daily during the 14-day post-treatment period. Clinical signs of toxic stress and body weights were recorded. Upon termination, rats were euthanized and gross pathology was performed.

Acute Inhalation Toxicity Limit Test

Five male and five female F-344 rats were exposed for 4 h to a target concentration of 5 mg/L of HFP/BP or PFB/BP. Exposures were performed using a nose-only inhalation chamber (Cannon et al., 1983). Animal body

weights were recorded prior to exposure and 1, 2, 4, 7, and 14 days post-dosing. Animals were observed twice daily during the postexposure period, and clinical signs of toxic stress were recorded. Rats were euthanized (CO_2 inhalation) and gross pathology performed on Day 14 postexposure.

Ames Test for Mutagenicity

Hexakis was examined for its potential to produce genetic toxicity using the Ames *Salmonella*/microsome mutagenesis assay (Brusick, 1994; Maron and Ames, 1983). Strains sensitive to frame-shift (TA98, TA1537) and base-pair substitution (TA100, TA1535) were used. Hexakis test doses were 0.3125, 0.625, 1.25, 2.5, and 5.0 mg/plate. Dimethylsulfoxide was used as a vehicle.

RESULTS

Details of results are available in the technical reports by Wolfe et al. 1997a, 1997b and Feldmann et al. 1997.

Oral Toxicity

Five male and five female rats were orally dosed with 5 g Hexakis/kg body weight. No deaths resulted from the oral administration of the test agent or vehicle, and no signs of toxicity were observed. All rats showed normal weight gains during the 14-day observation period (Tables 4.3-1 and 4.3-2). No gross lesions were observed at necropsy for any animals on study.

TABLE 4.3-1. BODY WEIGHTS[a] OF MALE F-344 RATS AFTER GAVAGE WITH 5 g HEXAKIS/kg BODY WEIGHT

Treatment	Animal Number	Days Post-treatment 0	1	2	4	7	14
Hexakis							
5 g/kg	M-06	245.9	255.6	257.5	254.1	258.5	269.4
5 g/kg	M-07	269.4	269.5	278.5	283.1	286.0	296.4
5 g/kg	M-08	250.5	253.7	254.7	255.2	258.5	267.8
5 g/kg	M-09	268.0	277.0	279.1	282.5	284.6	293.1
5 g/kg	M-10	267.7	271.6	279.1	279.4	283.0	286.3
	Mean	**260.3**	**265.5**	**269.8**	**270.9**	**274.1**	**282.6**
	SD	**11.2**	**10.3**	**12.5**	**14.9**	**14.3**	**13.3**
Control[b]							
1 mL/100 g	M-01	245.2	255.5	254.0	254.8	257.3	262.9
1 mL/100 g	M-02	261.6	269.4	271.2	270.8	273.9	281.8
1 mL/100 g	M-03	236.8	247.4	249.3	248.7	250.0	259.8
1 mL/100 g	M-04	272.5	284.9	286.2	280.4	288.0	291.2
1 mL/100 g	M-05	231.6	236.8	237.5	238.4	240.8	246.6
	Mean	**249.5**	**258.8**	**259.6**	**258.6**	**262.0**	**268.5**
	SD	**17.1**	**18.8**	**19.2**	**16.9**	**18.9**	**17.9**

[a]Weight in grams.
[b]1% agar solution.

TABLE 4.3-2. BODY WEIGHTS[a] OF FEMALE F-344 RATS AFTER GAVAGE WITH 5 g HEXAKIS/kg BODY WEIGHT

Treatment	Animal Number	Days Post-treatment 0	1	2	4	7	14
Hexakis							
5 g/kg	F-06	153.5	155.4	155.3	151.3	161.2	159.3
5 g/kg	F-07	154.5	162.7	162.0	166.8	169.8	174.4
5 g/kg	F-08	155.5	159.4	158.7	159.6	164.0	170.0
5 g/kg	F-09	164.8	171.3	175.7	175.9	181.5	184.9
5 g/kg	F-10	154.1	161.7	160.1	160.1	166.0	163.7
	Mean	**156.5**	**162.1**	**162.4**	**162.7**	**168.5**	**170.5**
	SD	**4.7**	**5.9**	**7.8**	**9.2**	**7.9**	**9.9**
Control[b]							
1 mL/100 g	F-01	150.1	152.5	153.4	154.6	161.3	162.8
1 mL/100 g	F-02	153.8	160.8	159.9	161.4	167.6	164.8
1 mL/100 g	F-03	157.6	168.0	165.9	171.5	173.8	172.1
1 mL/100 g	F-04	149.8	157.6	160.6	161.2	168.8	166.6
1 mL/100 g	F-05	153.9	157.5	156.4	153.7	153.0	149.3
	Mean	**153.0**	**159.3**	**159.2**	**160.5**	**164.9**	**163.1**
	SD	**3.2**	**5.7**	**4.7**	**7.1**	**8.0**	**8.5**

[a]Weight in grams.
[b]1% agar solution.

Mutagenicity Assay of Hexakis

Results of TA98, TA100, TA1535, and TA1537 are summarized in Table 4.3-3, where the data is expressed as the average (± SD) revertant number per plate calculated from triplicate plates. There was no dose-dependence or increase in the number of revertants at all five doses when compared with control in all four test strains.

Acute Inhalation Toxicity - HFP/BP

Five male and five female rats were exposed to the 9:1 HFP/BP mixture. The mean mixture concentration for the 4-h exposure was 5.09 mg/L (SD 0.17). The distribution of the two components based on area units was 89.5% HFC-236fa and 10.5% BP (Wolfe et al. 1997b). No deaths resulted from the acute inhalation exposure, and no signs of toxicity were observed postexposure. All male rats gained weight over the 14-day observation period (Table 4.3-4). Two of the five female rats gained weight, whereas the other three females lost weight during the postexposure observation period. No gross lesions were observed at necropsy for any animals on study.

Acute Inhalation Toxicity - HFP/BP

Five male and five female rats were exposed to the test material/air atmosphere for four hours. The mean concentration for the exposure was 5.3 mg/L (SD 1.2). Details of exposure concentration and analyses are

available (Feldmann et al. 1997). No deaths resulted from the acute inhalation exposure and no signs of toxicity were observed either during exposure or postexposure. All male rats and four of five female rats gained weight over the 14-day observation period (Table 4.3-5). One female lost weight during the postexposure observation period. No gross lesions were observed at necropsy for any animal on study.

DISCUSSION

In the oral toxicity evaluation of hexakis, no deaths or signs of toxic stress were observed in any of the animals dosed at the limit test value of 5 g/kg body weight. Body weights during the subsequent 14-day observation periods appeared unaffected by treatment. Under the conditions of the limit test, hexakis did not produce toxicity via the oral route of administration. When examined for its potential to produce genetic toxicity using the *Salmonella*/microsome mutagenesis assay (Ames assay), hexakis did not produce mutagenicity in the bacterial (*Salmonella*) system, and was therefore determined not to be genotoxic.

TABLE 4.3-3. MUTAGENICITY ASSAY OF HEXAKIS IN AMES TEST

Number of Revertants/Plate[a]

	TA98		TA100		TA1535		TA1537	
Treatment	**S9+**	**S9-**	**S9+**	**S9-**	**S9+**	**S9-**	**S9+**	**S9-**
Negative Control DMSO	28 ± 4.6	22 ± 4	113 ± 8.7	120 ± 3.2	29 ± 3	24 ± 1	7 ± 2	5 ± 2
Positive Control 2-AF	1870 ± 75	35 ± 2.3	1174 ± 75	154 ± 8				
SA						551 ± 5.5		
9-AA								12 ± 1.5
Hexakis (mg/plate)								
5.0	36 ± 1.2	33 ± 1.0	131 ± 21	140 ± 11	25 ± 5	23 ± 1.0	7 ± 2.6	5 ± 1.5
2.5	33 ± 1.1	28 ± 1.5	127 ± 7	120 ± 8	23 ± 1	24 ± 1.2	7 ± 1.7	6 ± 1.5
1.25	32 ± 1.5	26 ± 1.5	123 ± 9	138 ± 15	24 ± 2	28 ± 2.0	6 ± 2.0	8 ± 1.0
0.625	30 ± 2.8	26 ± 2.1	131 ± 11	132 ± 7	26 ± 3	25 ± 3.7	6 ± 1.5	4 ± 1.0
0.3125	34 ± 3.7	28 ± 1.0	121 ± 24	141 ± 12	26 ± 2	28 ± 2.6	5 ± 1.7	4 ±<0.1

[a]Mean ± SD.

TABLE 4.3-4. BODY WEIGHTS[a] OF F-344 RATS AFTER ACUTE INHALATION EXPOSURE TO 5 mg/L OF A 9:1 MIXTURE OF HFP/BP

Animal Number	Study Day 0	1	2	7	14
Male					
01	266.3	263.4	262.6	269.7	273.1
02	284.5	277.8	278.7	280.5	285.8
03	268.3	263.9	259.0	261.8	271.7
04	245.2	238.5	236.0	237.9	252.1
05	289.8	284.7	284.1	290.6	294.9
Mean	270.8	265.7	264.1	268.1	275.5
SD	17.5	17.7	18.9	20.1	16.2
Female					
01	175.8	171.5	173.6	173.6	177.7
02	180.7	176.7	178.4	179.7	183.5
03	171.0	170.2	168.0	169.5	170.9
04	183.5	180.3	178.2	179.0	176.7
05	163.8	156.6	155.4	152.7	154.5
Mean	175.0	171.1	170.7	170.9	172.7
SD	7.9	9.0	9.6	11.0	11.1

[a]Weight in grams.

TABLE 4.3-5. BODY WEIGHTS[a] OF MALE and FEMALE RATS AFTER ACUTE INHALATION EXPOSURE TO PFB/BP

Animal Number	Study Day 0	1	2	7	14
Male					
01	236.6	231.2	234.2	245.3	259.5
02	243.5	236.1	237.2	251.8	268.9
03	227.5	221.7	224.1	238.6	257.0
04	239.2	235.7	235.6	251.6	272.4
05	227.3	225.4	224.2	237.6	253.5
Mean	**234.8**	**230.0**	**231.1**	**245.0**	**262.3**
SD	**7.2**	**6.3**	**6.4**	**6.8**	**8.0**
Female					
06	134.5	130.3	133.1	136.6	142.6
07	127.5	133.9	134.0	136.5	143.2
08	156.8	153.3	155.8	157.5	167.5
09	148.1	145.6	142.7	148.1	147.2
10	144.2	143.3	143.6	148.1	151.0
Mean	**142.2**	**141.3**	**141.8**	**145.4**	**150.3**
SD	**11.5**	**9.2**	**9.2**	**8.9**	**10.2**

[a]Weight in grams.

DISCUSSION (continued)

In the inhalation toxicity studies with HFP/BP and PFB/BP, no deaths or signs of toxic stress were observed in any of the animals exposed at the limit test value of 5 mg/L. A mild decrease in body weight following exposure is common in inhalation exposure systems requiring animal restraint and is considered a consequence of animal stress. Under the conditions of the limit test performed in this laboratory, these test substance candidates do not demonstrate an acute toxicological hazard when administered by the inhalation route.

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4.4 REPRODUCTIVE TOXICITY SCREEN OF TRIFLUOROIODOMETHANE (CF_3I) IN SPRAGUE-DAWLEY RATS - A Final Report

D.E. Dodd, J.H. English[1], H.F. Leahy, M.L. Feldmann, and A. Vinegar

ABSTRACT

CF_3I is being considered by the U.S. Air Force as a replacement for Halon 1301 for fire-extinguishing requirements in unoccupied spaces. Previous acute and subchronic nose-only inhalation studies in Fischer 344 rats (Dodd et al. 1997) indicate minimal to mild toxicity at CF_3I concentrations of 2 to 8 % (v/v). The purpose of this study was to determine and evaluate the potential for CF_3I to produce reproductive toxicity and to provide additional information on the effect of CF_3I exposure on the thyroid. Groups of 16 male and 16 female rats were exposed (6 h/day) to CF_3I vapor at concentrations of 0 (control), 0.2, 0.7, and 2.0% using whole body inhalation chambers. Prior to mating, rats were exposed to CF_3I for 4 weeks (5 days/wk). Exposures were 7 days/wk during the periods of mating (2 wk), gestation (3 wk), and lactation (3 wk). First generation pups were not exposed to CF_3I vapor. In parental animals, there were no clinical signs of toxicity except for a mild decrease in mean body weight values at 2.0% CF_3I. At necropsy, gross findings, mean serum chemistry levels, mean hematology values, and mean organ weights were similar for all exposure groups, including the air control group. Analysis of reproductive indices and parameters indicate CF_3I is not a reproductive toxicant. Results of serum thyroid hormone levels (e.g., T_3, T_4, rT_3, and TSH), bone marrow evaluations for presence of micronuclei, and tissue histopathology are near completion. First generation pup survival and mean body weights were similar in all exposure groups, including the control. Based on data collected and analyzed to-date, exposure of 2.0% CF_3I vapor for more than 12 weeks produced minimal general toxicity and no reproductive toxicity in Sprague-Dawley rats. Supported by the Department of the Air Force, Contract No. F41624-96-C-9010.

INTRODUCTION

Environmental concern over the depletion of stratospheric ozone and global warming has led to an international treaty called the The Montreal Protocol which calls for the phase out of halons by the year 2000. Presently, the U.S. Air Force is using Halon 1301 (CF_3Br) as a flooding agent for extinguishing in-flight aircraft and electronic equipment fires and for fire extinguishment in confined spaces. Because it has less ozone-depleting activity and excellent fire suppression properties, trifluoroiodomethane (CF_3I) is being considered as a replacement of Halon 1301 for fire extinguishing requirements in unoccupied spaces.

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CF_3I has a high vapor pressure under ambient conditions (541 mm Hg at 25 °C), thus inhalation is a major route of exposure for persons in the workplace. Some information is available in the literature concerning CF_3I toxicity. A modified acute inhalation toxicity test was performed in which rats were exposed in a nose-only chamber to 12% CF_3I for 15 min (Ledbetter, 1993). Excess salivation was observed in the rats upon removal from the chamber; however, all rats appeared to be fully recovered by 2 h postexposure. A 15-min, nose-only inhalation study in rats determined the LC_{50} value to be 27% CF_3I (Ledbetter, 1994). As part of the process to develop environmental and health effects criteria, acute 2-wk and 13-wk nose-only inhalation toxicity studies were conducted in Fischer 344 rats (Dodd et al., 1997a). In the acute study, rats were exposed to 1.0, 0.5, or 0% (control) CF_3I for 4 h and sacrificed immediately following exposure, 3 days postexposure or 14 days postexposure. There were no deaths and no clinical signs of toxicity throughout the study. Histopathologic examination of select tissues showed no lesions of pathologic significance. In the 2-wk study, rats were exposed for 2 h/day, 5 days/wk to 12, 6, 3, or 0% CF_3I. No deaths were observed, though lethargy and slight incoordination were noted in rats of the 12 and 6% groups at the conclusion of each daily exposure. Mean body weight gains were depressed in rats of the 12% and 6% groups. Serum thyroglobulin and reverse T_3 (rT_3) values were increased at all exposure levels. At necropsy, no gross lesions or differences in absolute or relative organ weights were noted. Histopathologic examination of the thyroid and parathyroids indicated no morphological abnormalities in the CF_3I-exposed rats.

In the 13-wk study, four groups of 15 male and 15 female rats were exposed to 8, 4, 2, or 0% CF_3I 2 h/day, 5 days/wk for 13 wk. Rats exposed to 8 or 4% CF_3I had lower mean body weights than the controls. Deaths observed in the 2 and 8% groups were attributed to accidents resulting from the restraint system employed. Hematologic alterations were minimal and considered insignificant. Increases in the frequency of micronucleated bone marrow polychromatic erythrocytes were observed in rats of all three CF_3I groups. Serum chemistry alterations observed in rats of all CF_3I exposure groups included decreases in T_3 and increases in thyroglobulin, rT_3, T_4, and TSH. Relative organ weight increases (8% CF_3I group) occurred in the brain, liver, and thyroid; decreases were observed in the thymus and testes. A decrease in relative thymus weights and an increase in relative thyroid weights were observed also in rats of the 2 and 4% groups. Histopathological findings included a mild inflammation in the nasal turbinates of rats exposed to 4 or 8% CF_3I, mild atrophy and degeneration of the testes (4 and 8% CF_3I groups), and a mild increase in thyroid follicular colloid content in rats of all CF_3I exposure groups.

In genotoxicity testing protocols (Dodd et al. 1997b) CF_3I was positive with and without metabolic activation in the *Salmonella typhimurium* histidine reversion mutagenesis assay. The L5178Y/*tk* mouse lymphoma cell mutagenesis assay showed that CF_3I did not induce gene or chromosomal mutations in mammalian cells *in vitro*. However, a positive evaluation in the mouse bone marrow erythrocyte micronucleus test indicated that CF_3I was clastogenic *in vivo*. Cardiac sensitization testing of CF_3I vapor using beagle dogs showed a No Observable Effect

Level (NOAEL) at 0.2% and a Lowest Observable Adverse Effect Level (LOAEL) at 0.4% (Dodd and Vinegar, 1998). This toxicity precludes the use of this compound as a flooding agent in occupied spaces, but it may still be used in unoccupied spaces (Vinegar et al, 1995). Gas uptake kinetics of CF_3I have been studied (Williams et al., 1994), and a physiologically based pharmacokinetic model has been developed to simulate blood concentrations of CF_3I during inhalation exposures (Vinegar and Jepson, 1996).

Results from the repeated exposure mammalian studies and the genotoxicity tests suggest that additional studies are needed to complete a toxicity profile with CF_3I. No reproductive or developmental toxicity data are available. Further, a NOAEL has not been established for CF_3I in the rat.

Study Objective

The purpose of this investigation was to determine and evaluate the potential of CF_3I to produce alterations in parental fertility, maternal pregnancy and lactation, and growth and development of offspring in the rat. A NOAEL for CF_3I was to be determined. Additionally, efforts were made to answer the following questions posed by recently completed toxicity studies on CF_3I. Are the testicular effects that were observed in rats in the 13-week study transient, an artifact, or a reproductive hazard? Are the genotoxic effects that were observed in both *in vitro* and *in vivo* tests persistent at low exposure concentrations, and do they pose a genetic hazard? Are the thyroid effects that were observed in rodent subchronic studies persistent at low exposure concentrations, and do they pose a functional hormonal hazard? Exposure concentrations selected for this study extended beyond those of the 13-wk study and all target organs were thoroughly examined.

MATERIALS AND METHODS

Complete details of materials and methods are available in an Air Force technical report (Dodd et al., 1998).

Test Material

The test material, trifluoroiodomethane (CAS no. 2314-97-8), has a boiling point of -2.5 °C and a vapor pressure of 541 mm Hg @ 25 °C. The supplier of CF_3I test material was Ajay North America, LLC., Powder Springs, GA. The purity of the test material was determined by gas chromatography/mass spectrometry (GC/MS).

Laboratory Animals and Animal Husbandry

The Sprague-Dawley (SD) albino rat is the species of choice for reproduction studies because of high fecundity (EPA Health Effects Testing Guidelines, 40 CFR, section 798.4700). Seventy (70) male and sixty-seven (67) female (nulliparous and non-pregnant) Sprague-Dawley Crl:CD®(SD)BR rats, 8-9 weeks of age, were ordered and received from Charles River Breeding Laboratories, Hollister, CA.

Experimental Design

Group	No. Animals		CF_3I Concentration
	Male	Female	(%)
Control	16	16	0.0
Low	16	16	0.2
Middle	16	16	0.7
High	16	16	2.0
Positive control*	6	3	no exposure

***These rats were used only to serve as positive controls for the micronuclei in bone marrow erythrocytes assay.**

The animals were exposed to CF_3I vapor for four weeks (6 h/day, 5 days/week) prior to mating. The animals were mated for 14 days within their appropriate treatment level. During the mating, gestation, and lactation phase, animals were exposed 6 h/day, 7 days/week. However, dams were not exposed from Gestation Day 21 through Lactation Day 4 to allow for parturition and early lactation. Pups were not placed in exposure chambers, but remained in home cages separated from the dams for 6 h/day during Lactation Days 5 through 21. Following gestation Day 21 of the last female on study to deliver pups, exposure to CF_3I vapor (or air) returned to 6 h/day, 5 days/week, until the final day of termination for all remaining animals on study.

Test Material Generation and Analysis

Whole-body inhalation exposures were performed in 690-L chambers made of stainless steel and glass, similar in design to those described by Hinners et al 1968. The CF_3I and air for dilution were controlled through flow meters. Fine control of chamber concentration was made by minor adjustment of the CF_3I flow in response to chemical analysis of the chamber atmosphere. Total chamber air flow was approximately 60 L/min. Relative humidity and temperature of the exposure atmosphere were constantly monitored and recorded. Continuous analysis of the chamber air for CF_3I was performed using infrared absorption spectrometers. Instrumental calibration was performed using known concentrations of freshly prepared CF_3I in air contained in tedlar sample bags. Calibration checks were performed at appropriate intervals. Chamber atmosphere analyses of CF_3I were expressed in percent by volume or ppm (v/v).

RESULTS

Complete details of results are available in an Air Force technical report (Dodd et al., 1998).

Test Material Analysis, Chamber Atmosphere Analysis and Chamber Environment

The test material was 99.7+% CF_3I and remained stable throughout the study period. Results of the daily analytical and nominal mean concentrations of CF_3I, chamber temperature, and chamber relative humidity for each exposure group are given in Table 4.4-1.

Parental Animals

Clinical Observations and Body Weights

Rats were observed at least twice daily (morning and afternoon), including inhalation exposure periods. There were no treatment-related clinical findings. Areas of alopecia were sporadic and considered incidental. Mean body weights are given in Figures 4.4-1 (male rats) and 4.4-2 (female rats). Though not statistically significant, except for the final body weight values in female rats (Day 93-95), there was a marginal decrease in mean body weights of the 2.0% group compared to the control group. The mild decrease in absolute body weights for this group of rats is due primarily to a depression in body weight gain during the first 2-3 weeks of the study. Body weight values were normal for male and female rats of the 0.2 and 0.7% CF_3I groups.

TABLE 4.4-1. CHAMBER ATMOSPHERE ANALYSIS OF CF_3I AND CHAMBER ENVIRONMENT[a]

Target CF_3I Concentration (%)	Analyzed CF_3I Concentration (%)	Nominal CF_3I Concentration (%)	Analytical to Nominal Ratio	Chamber Temperature (°F)	Chamber Relative Humidity (%)
0	0.0	0.0		74.2 ± 1.6	54.8 ± 6.5
0.2	0.20 ± 0.01	0.24 ± 0.01	0.83	73.9 ± 1.8	43.4 ± 5.2
0.7	0.71 ± 0.01	0.72 ± 0.03	0.99	74.1 ± 1.7	50.5 ± 5.0
2.0	2.02 ± 0.03	2.08 ± 0.09	0.97	73.5 ± 1.6	54.8 ± 6.5

[a]**Values are reported as mean ± SD of daily means where appropriate.**

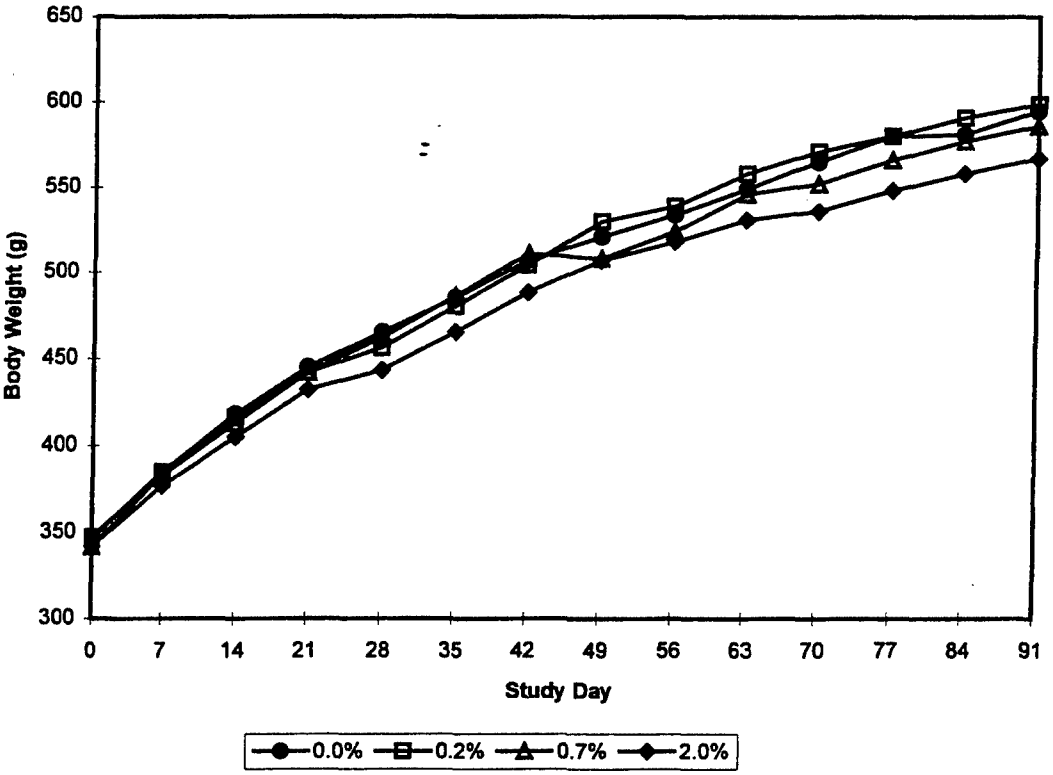

Figure 4.4-1. Mean Body Weights of Male Rats Exposed to CF_3I.

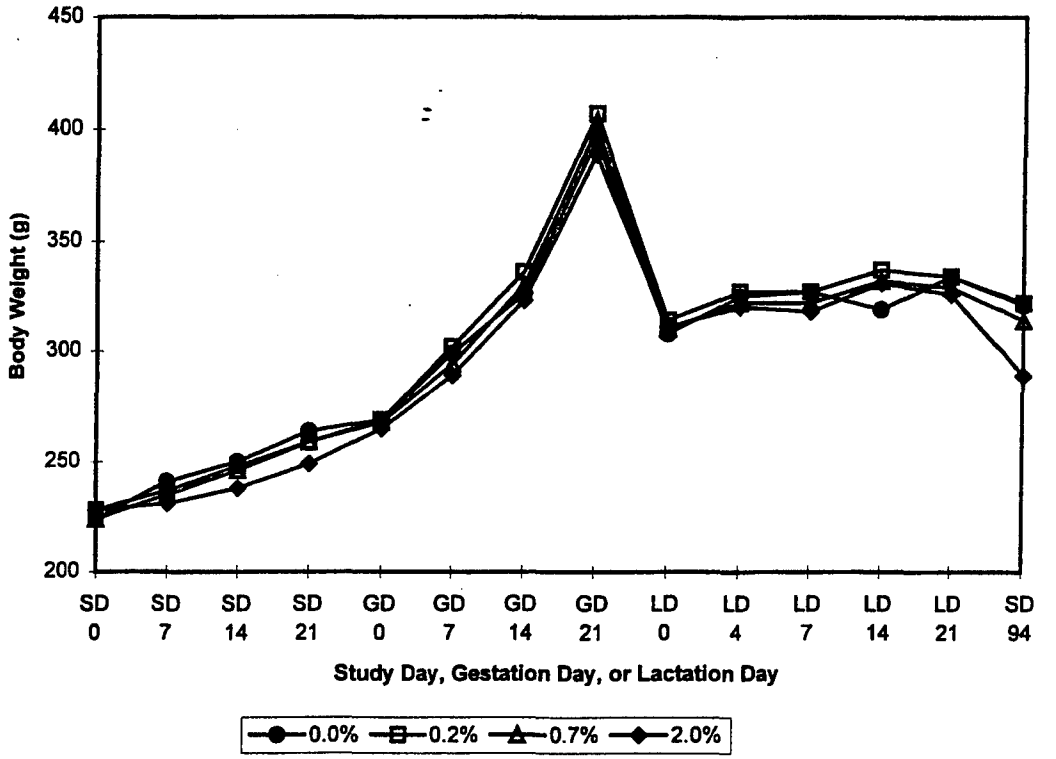

Figure 4.4-2. Mean Body Weights of Female Rats Exposed to CF_3I.

Clinical Pathology - Hematology and Serum Chemistry

For male rats, statistically significant differences in hematology and serum chemistry parameters were few in number and not considered treatment related due to: lack of consistency with time (7-wk compared to 14-wk values), lack of dose-response, or the difference was of small magnitude and not considered biologically important. For female rats, statistically significant differences in hematology and serum chemistry parameters were not considered CF_3I exposure related for the same reasons stated above. Though mild increases in cholesterol, total protein, and albumin were observed in both the 0.7 and 2.0% CF_3I groups of female rats, a concentration-related increase was lacking.

Clinical Pathology - Serum Thyroid Hormones

Serum thyroid hormone values for male rats after 7-wk exposure to CF_3I are given in Table 4.4-2. Table 4.4-3 has thyroid hormone levels for male and female rats after 14-wk exposure to CF_3I. Statistically significant and concentration-related increases in T_4 and rT_3 were observed in male and female rats after 7 or 14 wks of exposure. T_3 levels were decreased in a concentration-related manner in these animals. For TSH, an increase was observed in CF_3I-exposed male rats after 7-wk exposure, but there was no concentration relationship. After 14-wk exposure, male and female rats of the 2.0% group had statistically significant increases in TSH levels compared to control rats. A marginal and statistically significant increase in TSH was observed in female rats of the 0.2% group, but this effect was not statistically significant in female rats at 0.7% CF_3I.

Micronuclei in Bone Marrow Erythrocytes

Micronuclei scores for male and female rats exposed to CF_3I for 7 or 14 wk were similar to micronuclei scores in the control animals. Positive control animals had mean micronuceli scores that were two- to five-fold higher than negative control values. The weak response in the positive control animals was due to the administration of a small dose of cyclophosphamide to induce micronuclei formation. The ratio of PCE/NCE (an indicator of bone marrow cell toxicity) was similar in all study groups, control or CF_3I exposure.

Reproductive Data

Results of the reproductive assessment are given in Table 4.4-4. There were no statistically significant differences between CF_3I-treated and control groups in all indices and endpoints measured, except for a low pup sex ratio (males:females) in the 2.0% group.

Gross Necropsy and Organ Weights

There were no clinically significant or CF_3I-exposure related gross lesions in any of the study animals. Statistically significant differences from the organ weight means of the control animals follow. Relative liver weights were mildly increased (6-10% above control values) in the male rats of the 2.0% group at both the 7-wk and 14-wk sacrifice periods. Mean absolute and relative epididymides weights were also increased in male rats of the 0.7 and 2.0% groups at 7 wk, but not at 14 wk. The increase in epididymides weights was not CF_3I-

concentration dependent. For female rats, decreases in mean absolute organ weights included the brain (0.7 and 2.0% CF_3I), ovaries (0.7 and 2.0%), and heart (2.0%). Mean relative brain (2.0%), kidney (2.0%), and liver (0.7%) weights were increased. Mean relative ovary weights were decreased (0.7 and 2.0% CF_3I). Note that mean body weight was statistically significantly decreased in the 2.0% female rats compared to the control female rats.

TABLE 4.4-2. SERUM THYROID HORMONE VALUES[a] FOR MALE RATS EXPOSED TO CF_3I FOR 7 WEEKS

Parameter	0.0% CF_3I	0.2% CF_3I	0.7% CF_3I	2.0% CF_3I
TSH(ng/mL)	3.58 ± 0.30	5.50[d] ± 0.55	5.96[d] ± 0.42	5.94[d] ± 0.54
T_4 (µg/dL)	3.46 ± 0.31	5.48[d] ± 0.51	7.56[d] ± 0.80	8.74[d] ± 0.82
T_3 (ng/dL)	138 ± 11	121[b] ± 12	113[d] ± 8	108[d] ± 9
rT_3 (ng/dL)	4.76 ± 0.45	6.79[d] ± 0.49	7.56[d]± 0.73	14.5[d]± 1.1

[a]Mean ± SD, n=7 to 9.
[b]p<0.05 compared to control.
[c]p<0.01 compared to control.
[d]p<0.001 compared to control.

TABLE 4.4-3. SERUM THYROID HORMONE VALUES[a] FOR MALE AND FEMALE RATS EXPOSED TO CF_3I FOR 14 WEEKS

Parameter	Sex	0.0% CF_3I	0.2% CF_3I	0.7% CF_3I	2.0% CF_3I
TSH(ng/mL)	M	3.77 ± 0.33	3.66 ± 0.40	3.87 ± 0.40	6.60[d] ± 0.68
	F	6.60 ± 0.75	7.99[b] ± 0.82	7.61 ± 0.81	17.96[d] ± 1.91
T_4 (µg/dL)	M	3.80 ± 0.35	5.32[d] ± 0.56	7.28[d] ± 0.76	7.80[d] ± 0.56
	F	4.09 ± 0.46	7.50[d] ± 0.78	8.91[d] ± 1.00	10.43[d] ± 1.06
T_3 (ng/dL)	M	143 ± 8	107[d] ± 12	115[d] ± 10	105[d] ± 10
	F	169 ± 17	145[d] ± 16	125[d] ± 15	107[d] ± 11
rT_3 (ng/dL)	M	4.55 ± 0.53	7.43[d] ± 0.79	9.03[d]± 0.90	13.1[d]± 1.28
	F	6.40 ± 0.70	11.1[d] ± 1.14	14.4[d]± 1.30	25.7[d] ± 2.23

[a]Mean ± SD, n=15 or 16 for females, 8 for males.
[b]p<0.05 compared to control.
[c]p<0.01 compared to control.
[d]p<0.001 compared to control.

TABLE 4.4-4. REPRODUCTIVE DATA FOR RATS EXPOSED TO CF_3I FOR 14 WEEKS

Parameter	0.0% CF_3I	0.2% CF_3I	0.7% CF_3I	2.0% CF_3I
No. of Females Paired (placed with a male)	16	16	16	16
Mating Index[a] (%)	100	94	94	81
Fecundity Index[b] (%)	94	80	100	85
Fertility Index[c] (%)	94	75	94	69
Mean[d] Gestation Length (days)	22.2 ± 0.7	22.4 ± 0.5	22.2 ± 0.4	22.6 ± 0.5
Gestation Index (%)[e]	100	100	100	91
Mean[d] No. of Pups per Litter	13.3 ± 4.2	12.5 ± 4.6	14.7 ± 2.3	11.6 ± 3.9
Pup Sex Ratio[f]	0.99	0.79	1.07	0.68[k]
Live Birth Index[d,g] (%)	100 ± 0	99 ± 5	99 ± 3	90 ± 30
4-Day Survival Index[d,h] (%)	99 ± 2	100 ± 0	98 ± 3	98 ± 3
7-Day Survival Index[d,j] (%)	100 ± 0	97 ± 11	98 ± 4	100 ± 0
14-Day Survival Index[d,j] (%)	100 ± 0	97 ± 11	98 ± 4	100 ± 0
21-Day Survival Index[d,j] (%)	100 ± 0	97 ± 11	98 ± 4	100 ± 0

[a] $\frac{\text{Number of females with plug or sperm positive x 100}}{\text{Number of females paired}}$

[b] $\frac{\text{Number of females delivering a litter x 100}}{\text{Number of females with plug or sperm positive}}$

[c] $\frac{\text{Number of females delivering a litter x 100}}{\text{Number of females paired}}$

[d] Mean ± SD

[e] $\frac{\text{Number of females with live litters x 100}}{\text{Number of females delivering a litter}}$

[f] $\frac{\text{Number of male pups per group}}{\text{Number of female pups per group}}$

[g] $\frac{\text{Number of live pups at birth x 100}}{\text{Number of pups born}}$

[h] $\frac{\text{Number of pups surviving 4 days x 100}}{\text{Number of live pups at birth}}$

[j] $\frac{\text{Number of pups surviving 7-,14-,or 21 days x100}}{\text{Number of pups retained at 4 days}}$

[k] $p < 0.05$ compared to control

Histopathology

There were no lesions of clinical significance in any CF_3I-treated (2.0% group) or control group animals. Tissues from animals in the 0.7 or 0.2% CF_3I groups were not examined microscopically due to the lack of histopathologic findings in rats of the control and 2.0% CF_3I groups.

Progeny

Clinical Observations and Pup Weights

There were no CF_3I-treatment related clinical observations in the first generation pups from birth to postnatal Day 21. Pup survival indices are given in Table 4.4-5. There were no statistically significant differences between control and CF_3I exposure groups in mean pup survival indices. There were no statistically significant differences between control and CF_3I exposure groups in mean pup weights.

DISCUSSION

This study met its objective, that is, to determine and evaluate the potential of CF_3I to produce alterations in parental fertility; maternal pregnancy and lactation; and growth and development of offspring in the rat. A No Observable Effect Level (NOEL) is recommended (below). Additionally, questions raised on the effects of CF_3I exposure following the recently completed acute and subchronic inhalation toxicity studies (Dodd et al., 1997a) were answered.

A brief synopsis of the results of this study and interpretation, where necessary, follows. In parental animals, there were no clinical signs of toxicity. A minimal decrease in mean body weight in rats of the 2.0% CF_3I group was observed, though the decrease was not statistically significant, except at the conclusion of the study (female rats only). Statistically significant differences in hematology and serum chemistry parameters (except thyroid hormones) were few in number and not considered treatment related or biologically important due to lack of consistency with time (7-wk compared to 14-wk values), lack of dose-response, or the difference was of small magnitude. Statistically significant, concentration-related increases in serum TSH, T_4, and rT_3 were observed. T_3 levels were decreased. The alterations in serum thyroid hormones caused by CF_3I exposure were similar to those observed previously in this laboratory (Dodd et al., 1997a), and provide further support for the mechanistic theory of inhibition of 5'-deiodinase, an enzyme that catalyzes the conversion of T_4 to T_3. Reduced levels of T_3 initiates an increase in TSH. Increases in TSH lead to increases in T_4. When the conversion of T_4 to T_3 is inhibited, increases in rT_3 occur. Though there are toxicity concerns, such as thyroid goiter and tumor development, for chemicals that induce a sustained increase in the secretion of pituitary TSH, rats and mice are highly sensitive to these effects compared to humans (Capen, 1995; McClain et al., 1988, 1989). Humans can have markedly altered changes in thyroid function and elevated TSH levels, as in areas with a high incidence of endemic goiter because of iodine deficiency, but there is little, if any, increase in the incidence of thyroid cancer (Curren and DeGroot, 1991). Thresholds for a "no-effect" level on the thyroid gland can be established by determining the

dose of xenobiotic that fails to elicit an elevation in the circulating level of TSH (Capen, 1996, personal communication). In rodent studies, this "no effect" level would provide a wide margin of safety for danger to the human thyroid due to considerable differences in thyroid hormone economy and response of follicular cells to TSH between rodents and man (Capen, 1995, 1996, personal communication).

In the current investigation, there was no increase in micronuclei frequency in the bone marrow erythrocytes of rats exposed to CF_3I. Though CF_3I was positive in the Ames and mouse bone marrow erythrocyte tests (Dodd et al., 1997b), and induction of micronuclei was observed in rats exposed to CF_3I at concentrations ranging from 2 to 8% (Dodd et al., 1997a), the effect observed previously at 2% CF_3I is not reproducible. Additionally, the PCE/NCE ratio, an indicator of bone marrow toxicity, was unaltered in the current study. In the previous study (Dodd et al., 1997a), the PCE/NCE ratio decreased indicating toxicity by CF_3I exposure. The difference in results of micronuclei scores and PCE/NCE ratios between the previous and current studies suggests that stress due to nose-only inhalation exposure confinement (previous study) may be a factor. Strain difference (Fisher 344 versus Sprague-Dawley CD) might also be a factor. Also, it is general knowledge that the dose of a test agent varies in animals when different inhalation exposure delivery systems (nose-only versus whole-body) are used, though the exposure concentrations for each delivery system are the same. Nose-only systems generally involve higher rates of respiration because of the added stress of this route of exposure as compared with whole-body. This would result in a higher accumulative dose for animals exposed in a nose-only system.

In the current investigation, there were no treatment-related gross lesions at necropsy. Statistically significant differences in mean organ weights were few in number and not considered biologically important for the same reasons as cited above. CF_3I exposure did not cause microscopic changes in tissues and organs, including the target organ, the thyroid. Analysis of reproductive indices and parameters, including pup survival and growth, indicate CF_3I is not a reproductive toxicant. This analysis included statistical analyses of the current reproductive data and comparison of historical reproductive indices in control rats in five reproductive toxicity screens conducted at this laboratory from 1991-1996. Testicular lesions observed in rats exposed to 4 or 8% CF_3I (Dodd et al., 1997a) may have been a result of heat stress, due to animals being placed in restraining tubes during the inhalation exposure regimen.

In conclusion, exposure of 2.0% CF_3I vapor for approximately 14 weeks produced minimal general toxicity and no reproductive toxicity in Sprague-Dawley rats. On the basis of general toxicity, reproductive toxicity, and serum TSH concentrations in the current study and in previous studies on CF_3I (Dodd et al., 1997a, 1997b), the No Observable Effect Level (NOEL) is 0.7% CF_3I.

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SECTION 5

EXPLOSIVES, PROPELLANTS, FUELS, AND LUBRICANTS PROJECT

5.1 DERMAL ABSORPTION OF MODULAR ARTILLERY CHARGE

J.N. McDougal, K.O. Yu[1], D.T. Tsui[1], H. Zhang, D.L. Pollard, and G.W. Jepson

ABSTRACT

Soldiers handling Howitzer cartridges may have the potential to absorb the components of solid propellants through the skin of the hands. The purpose of this research was to determine if systemic absorption of chemical components of the new Modular Artillery Charge System (MACS) might be a hazard in operational military environments. We sampled the surfaces of solid propellant cartridges (XM232), which were stored under extreme environmental conditions, to determine the concentrations of various propellant components (nitroglycerin, diphenylamine, nitroguanidine, and nitrocellulose). After eleven months of storage, the only propellants found on the surface of the cartridges were very small quantities of nitroglycerin and diphenylamine. We used excised rat skin in static diffusion cells to investigate the dermal absorption of these components from the MACS in powdered form. Measured surface concentrations and fluxes from the diffusion studies were used to estimate systemic absorption in human exposure scenarios. Systemic absorption via the dermal route was compared with the American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Values (TLVs) for these chemicals. Our results suggest that these propellant components are not absorbed sufficiently to be a systemic hazard under anticipated use conditions.

INTRODUCTION

The Army Surgeon General's Office is charged with assessing the potential health hazards of new Army weapon systems. They are concerned about the potential for dermal absorption of solid propellants from the Modular Artillery Charge System (MACS). These propellants are fairly complex mixtures containing up to a dozen chemical components, including nitroglycerin, diphenylamine, nitroguanidine, and nitrocellulose. The systemic toxicity of MACS in female rats was recently investigated (Kinkead et al., 1995, 1996). After 28 days of being fed 2g-propellant per kilogram of rat, the only effect was Methemoglobin elevation. There were no effects on body weight, organ weight, or reproductive parameters. Dermal absorption measurements are required to be able to determine if continued dermal contact with the propellants and propellant casings is a systemic hazard. We are working with the artillery program manager at Picatinny Arsenal to address these and other toxicity questions prior to the approval of these new weapon systems. The purpose of this effort was to use fluxes from laboratory studies with excised rodent skin and measurements of XM232 component concentrations on the surface of the propellant casing to estimate the potential hazard to soldiers.

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Propellants

The XM232 propellants are one of the improved solid charges which will replace current 155mm Howitzer propellants. These propellants are completely encased in a rigid combustible case and are placed in the cannon (one to five charges) along with the projectile at the time of firing. Each charge increment contains additives to reduce flash, wear, and coppering. Range is adjusted by the number of increments which are placed in the Howitzer. Chemical substances of concern are those comprising the rigid combustible case, the propellant, and combustion product residues which remain on the gun surfaces after firing (Army Environmental Hygiene Agency, 1994).

Primary components of the XM232 propellant are: nitrocellulose (33%); nitroguanidine (39%); nitroglycerin (19%); potassium sulfate (1%); Ethyl Centralite (1%); resin (1%); Kraft fiber (1%); and bismuth oxide (1%). Primary components of the combustible case are Nitrocellulose (72%); Kraft fiber (17%); Resin & additives (10%); diphenylamine (1%) and others (4%). The ACGIH has only determined TLVs for nitroglycerin (0.46 mg/m3), diphenylamine (10 mg/m3), and graphite (2 mg/m3) (ACGIH, 1996). Nitroglycerin also has a skin notation which means that there is a "potential significant contribution to the overall exposure by the dermal route." There is no information about the dermal absorption of these chemicals except nitroglycerin, which has been studied from many different types of vehicles. Nitroglycerin is used therapeutically in transdermal delivery devices for angina, so it obviously can be formulated to penetrate the skin.

Surface Sampling

The surfaces of twelve XM232 Modular Artillery Charge Shells were sampled at Yuma Air Station in May 1997 after being stored outside in metal canisters for eleven months. These canisters were exposed to the extreme temperature fluctuations which are normal in the desert during a year (30 °F to 119 °F and 10% to 100% relative humidity). The exterior surface of the cell was sampled by carefully wiping one quarter of the cylindrical surface of the charge (280 cm2) with a dry gauze pad. Samples were transferred to capped centrifuge tubes and carried back to Wright-Patterson AFB for analysis by HPLC with ultraviolet detection. These samples were analyzed for nitroglycerin, diphenylamine, nitroguanidine, nitrocellulose, and 4-aminobiphenyl (a potential contaminant of diphenylamine).

Static Diffusion Cells

In these *in vitro* experiments, static diffusion cells are used to measure the flux of chemical across the skin or a membrane. Diffusion cells come in many configurations and sizes, but the general principle is the same. Figure 3.1-1 shows a schematic depiction of a common diffusion cell. The outer surface of the skin is placed between a donor and receptor cell and clamped in place. The receptor cell is surrounded by a water jacket through which warm water circulates. Powder is placed on the outer surface of the skin and the appearance of chemical in the

receptor solution is determined, over time, by taking samples of the receptor solution. The receptor solution is well-stirred and care is taken to keep air out of the system so that the skin is in complete contact with the receptor solution. Chemical concentration is usually analyzed using gas chromatography, high performance liquid chromatography, radiochemical analysis, or other methods.

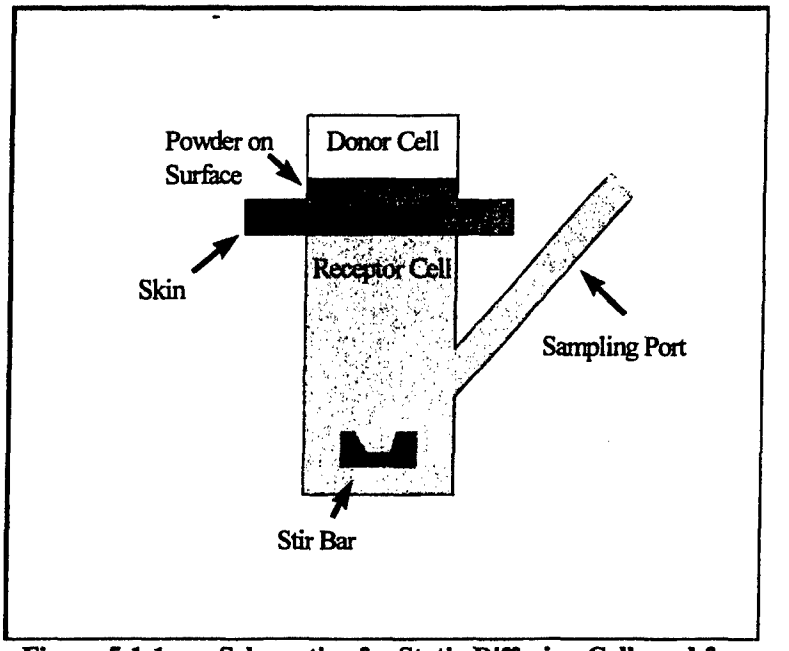

Figure 5.1-1. Schematic of a Static Diffusion Cell used for Flux Measurements

Flux and Skin Absorption Time

Flux is determined from the slope of the cumulative flux versus time plot, and traditionally has units of mass/(area x time). The factors which impact steady-state flux are illustrated in a version of Fick's Law (Scheuplein and Blank, 1971):

$$Flux = \frac{K_m D}{\delta}(C_{out} - C_{in})$$

where Km is the membrane/chemical partition coefficient; D is diffusivity (length2/time); δ is thickness (length) of the membrane; Cout is concentration (mass/length3) outside the membrane; and Cin is concentration inside the membrane.

Skin absorption time (SAT) is a new method to relate potential dermal exposures to existing guidelines such as the ACGIH Threshold Limit Values (TLVs) or OSHA's Permissible Exposure Level (PEL) (Walker et al., 1996). This method has been developed for pure liquids or liquid solutions, but we have modified it here, for powders or solids. It requires absorption rate information and provides quantitative information that is both intuitive and

useful in a case such as this. It is a two step process: First, an estimate of the total amount of chemical which would be inhaled at the occupational limit value is calculated:

$$TA = EL \times RR \times T$$

where;

TA is total chemical absorbed (mg),
EL is the exposure limit (mg/m3),
RR is the appropriate respiratory rate (m3/h), and
T is the shift time (h).

Then, the time required to get this amount of chemical through the skin is calculated:

$$SAT = \left(\frac{TA}{MD \times k \times A} \right)$$

where;

SAT is skin absorption time (h),
MD is the mass density of the chemical to which the skin is exposed (mg/cm2),
k is a first order rate (h-1) related to flux, and
A is surface area of skin exposed (cm_2).

The denominator of Equation 3, which calculated the total absorbed from the skin route, is novel but intuitive. Amount of chemical diffusing across the skin is proportional to the concentration difference across the skin, according to Equation 1. Mass density can be used as a surrogate for concentration, since concentration of a powder is hard to define. A change in mass density should also affect chemical absorption proportionally just like a change in concentration. When the amount of chemical per surface area on the skin is continually increased, amount diffusing across the skin would increase only up to a point. Past this point a further increase in mass on the skin causes no increase in flux; but below this point increasing mass density would cause a proportional increase in amount of chemical absorbed. In this regime, flux can be expressed as a pseudo first-order process, units of h-1, during the time before there is excess on the skin, analogous to the situation with concentration. Our experiments were done with excess MACS on the skin (500 µg/4.9 cm2) and, therefore, the first order rate that we calculated would be the maximum rate possible. Surface area exposed also proportionally affects the amount of chemical which penetrates, i.e., doubling the surface area doubles absorption if the other parameters stay the same. These equations make several assumptions:

the flux is constant throughout the whole exposure;
the mass of chemical on the skin is equivalent to the amount of chemical on the surface in contact with the hands; and
the mass of chemical on the exposed skin surface is constant (i.e., not diminished by absorption or accumulated by repeated contact).

Because of the assumptions and potential variability in this time calculation, it should not be interpreted to be a quantitative answer. The resultant SAT gives a categorical idea of whether it takes minutes, hours, or days to get the same body burden as one would get from being exposed to the limit value for a whole work shift.

RESULTS and DISCUSSION

Propellant on Shell Surfaces

Amount of components of XM232 on the surface of shells is shown in Table 5.1-1. Only nitroglycerin, nitrocellulose, and diphenylamine were found on the surface of the paper cartridge. It is not known whether these concentrations are left over from the manufacturing process or due to diffusion from the inside through the paper case to the surface. Nitroglycerin has been shown to diffuse through the paper casing of another shell (Manning, 1986). Diphenylamine is a minor component of the paper casing and may be available on the surface of the cartridge.

TABLE 5.1-1. MASS DENSITY OF CHEMICAL COMPONENTS OF XM232 CARTRIDGES FOUND ON THE CARTRIDGE SURFACE WITH A GAUZE WIPE

Chemical	Mass recovered (ug)	Mass density (ug/cm2)
Nitroglycerin	35.2	0.127
Diphenylamine	7.99	0.029

Our limits of detection for the other chemicals were 2.8 μg for nitrocellulose, 2.1 pg for nitroguanidine, 4.8 pg for 4-aminobiphenyl. We also used methanol-soaked gauze to wipe a different quadrant of the same cartridges. Because methanol is a good solvent for these propellants the methanol soaked gauze removed up to ten times more nitroglycerin from the surfaces of the cartridges. It appeared that the methanol wipes may have been desorbing chemicals from inside the paper casings instead of from the surface. We believe that dry gauze is a much better surrogate to estimate transfer to soldier's hands.

Dermal Absorption of Propellant in Rats

We attempted to measure the absorption of nitrocellulose, nitroguanidine, and nitroglycerin through excised rat skin in the static diffusion cell after placing powdered propellant on the surface of the skin, as previously described. Only nitroglycerin could be detected in the saline receptor solution; the other chemicals did not penetrate well enough to be above the limit of detection. The results of a diffusion cell experiment showing diffusion of nitroglycerin in six static diffusion cells is shown in Figure 5.1-2.

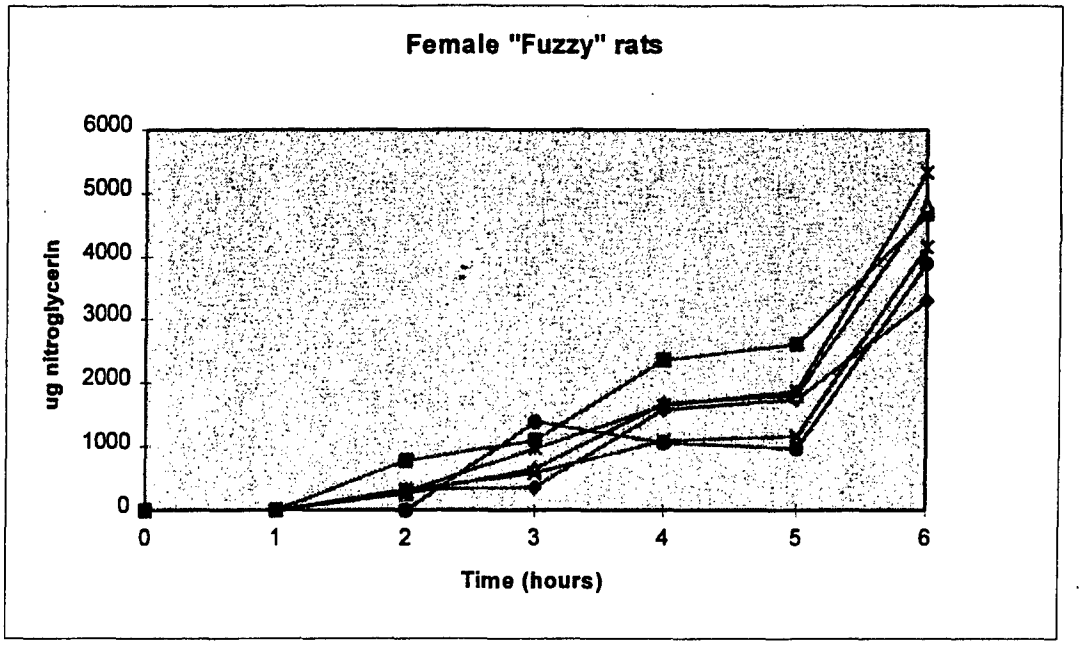

Figure 5.1-2. Plot of the cumulative amount of nitroglycerin absorbed through the excised skin of a female "Fuzzy" rat

The average nitroglycerin flux calculated from the slope of these plots was 0.15 μg/cm^2/h. The slope of a natural log plot of these data gives a value of 0.480 h-1. No flux values could be determined for the other two chemicals because they could not be detected in the receptor solution, but based on the detection limits, the flux for nitroguanidine and nitrocellulose must be three-to-five orders of magnitude less than the flux of nitroglycerin.

Skin Absorption Time

Nitroglycerin has a fairly low TLV (0.46 mg/m3), which when multiplied by OSHA's default respiratory rate for light work (10 m3 for an 8-h shift), gives an estimate of the total absorbed dose of 4.6 mg if exposed to the TLV for an entire work shift (see Equation 2). Based on Equation 3, we can calculate the skin absorption time for nitroglycerin absorption from XM232 using the palm area of both hands (500cm^2):

$$\frac{4.6\ mg}{0.000127\ mg/cm^2 \times 0.48\ hr^{-1} \times 500\ cm^2} = 151\ h$$

With the measured amounts of nitroglycerin on the surface of the cartridge and with the flux determined in the laboratory, it would take 151 hours of constant absorption across the skin of both hands for a soldier to absorb what is considered a "safe" amount of nitroglycerin by the ACGIH. Obviously, with only 24 hours in a day and constant exposure to this amount of nitroglycerin on the cartridge surface, we estimate that the maximum body burden would be one-sixth of the amount absorbed at the ACGIH TLV.

This value assumes that rat and human skin have the same permeability. Several studies have shown that rat skin is two-to-three times more permeable than human skin (Vecchia, 1997; McDougal, 1990). Our estimate of the amount absorbed would, therefore, err on the high side. This calculation also assumes that the health effects for which the TLV is protective are also the most important effects via the skin route. In the case of nitroglycerin, there appear to be no route-specific effects. Nitroglycerin affects smooth muscle structures, particularly in the vascular system, and the effects are severe headaches, dizziness, and postural weakness (Needleman and Johnson, 1980).

SUMMARY and CONCLUSIONS

The Army Surgeon General's office has tasked developers of new Howitzer systems to provide information about the dermal absorption of propellant residue on cartridges. With the Army RD&E Center at Picatinny Arsenal, we have developed a program to answer these questions. We measured amounts of propellants on the surface of canisters stored for eleven months, determined the flux of propellant components from powdered propellant across rat skin in the laboratory, and combined this information to give a temporal relationship between dermal exposures and acceptable inhalation exposure levels.

Of the five primary components measured on the surface of Howitzer cartridges stored for eleven months, only nitroglycerin and diphenylamine could be detected. All amounts on the surface were extremely low. It is not known for sure whether these chemicals were left on the surface during the manufacturing process, or whether they have diffused through the combustible paper casing. It is most likely that they have diffused through the casing since nitroguanidine (which is present in the propellant at about twice the concentration of nitroglycerin) was not found on the surface. We would expect all components of the propellant to be found on the surface if residue was left on the surface during manufacturing.

When powdered propellant was placed on rat skin in a standard diffusion cell in the laboratory, only nitroglycerin was found in the receptor solution. The nitroglycerin component of MACS penetrates rat skin at an extremely low rate. The nitroglycerin in transdermal drug-delivery systems is made to transfer more rapidly by proprietary formulations which enhance penetration. The other two major components of MACS, nitrocellulose and nitroguanidine, did not penetrate. Based on the good detection limit which we achieved for these chemicals, we think they can be ruled out as a dermal absorption hazard.

In conclusion, systemic absorption through the skin of the chemical components of this family of MACS powdered propellants would probably not be a hazard in operational military environments. We found that in nearly a year of storage at very warm temperatures, only nitroglycerin (component of the propellant) and diphenylamine (component of the combustible casing) could be found on the shell surface in any reasonable amounts. We also found that when rat skin was exposed to the powdered propellant mixture, only nitroglycerin

penetrated well enough to be detected. We expect that nitroglycerin would be the only potential hazard. The estimated length of time to exceed the acceptable (according to the TLV) body burden for nitroglycerin through the palmar surface of both hands, exposed to Howitzer shells such as these, would probably be much longer than an 8-h work shift. Based on our studies and calculations, we think that it is extremely unlikely that XM232 would constitute toxicological hazard to soldiers by dermal absorption.

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SECTION 6

PREDICTIVE TOXICOLOGY PROJECT

6.1 INTERLINKED PHARMACODYNAMIC MODEL FOR TRICHLOROETHYLENE (TCE) INDUCED OXIDATIVE STRESS

J.Z. Byczkowski

ABSTRACT

To provide a useful tool for risk characterization, a physiologically based pharmacodynamic (PBPD) interlinked model describing pro-oxidant effects of trichloroethylene (TCE) within the target organ was constructed in a way compatible with a physiologically based pharmacokinetic (PBPK) model which describes the internal dose of TCE. The focus of this research was on the quantitative description of oxidative stress in mammalian systems. Based on the available literature and our own experimental data, three basic modes of pro-oxidant action of TCE were modeled and simulated: (1) lipid peroxidation (as measured by the formation of TBARS in liver and the exhalation of ethane); (2) a single-hit interaction of free radicals with cellular targets; and (3) a stochastic multi-hit interaction of free radicals with multiple cellular targets. Based on the dose-response characteristics observed *in vitro*, a mathematical model of chemically initiated oxidative stress was developed and calibrated in B6C3F1 mice treated with TCE *in vivo*, using ethane exhalation as a measured end point. The resultant interlinked model may be used for description of oxidative stress in mice and potentially may be useful for risk characterization (Supported in part by Department of the Air Force Contract No. F41624-96-C-9010).

INTRODUCTION

Health risk associated with exposure to chemicals depends on both the extent of exposure (dose) and the mode of action of a chemical (response). For a quantitative modeling of the mode of action it is necessary to determine the exact chain of events of chemical interaction with the biophase and the local concentration of the chemical in the biophase. However, this task may be relatively simple only for those chemicals that have a very specific, single mechanism of action (for example, a specific inhibitor of a single enzyme). Pro-oxidant chemicals usually have multiple targets and produce diverse biological effects depending on the dose and type of tissue as well as the level of antioxidant protection.

In this report, a predictive tool potentially useful for risk characterization of a weak pro-oxidant chemical, trichloroethylene (TCE) is described. A quantitative, physiologically based pharmacodynamic (PBPD) model, describing biological effects within the target organ was constructed in a way compatible with pharmacokinetic (PBPK) model which describes the internal, local dose of TCE. Based on the available literature and our own experimental data, the three basic modes of action of pro-oxidant chemicals were modeled and simulated *in silico*: lipid peroxidation (expressed by the formation of TBARS in the liver and the exhalation of ethane); a single-hit interaction of free radicals with cellular targets; and a stochastic multi-hit interaction of free radicals with multiple cellular targets. Based on the dose-response characteristics verified *in vitro* (described by Byczkowski et al.,

1995), a PBPD module for chemically initiated oxidative stress was developed and calibrated in B6C3F1 mice treated with TCE *in vivo*, using the ethane exhalation as a measured end point (described by Byczkowski and Seckel, 1996). The developed PBPD module has been linked with a physiologically based pharmacokinetic (PBPK) module which described local concentrations of pro-oxidant chemicals in the liver (described by Byczkowski et al., 1997a). The resultant PBPD model may be used for a pharmacodynamic description of oxidative stress in mice and potentially may be useful for risk characterization (Byczkowski et al., 1997b).

MATERIALS AND METHODS

Model Description

The PBPD model (Figure 6.1-1) was written in the Advanced Continuous Simulation Language (ACSL©) and simulations were performed using SIMUSOLV software on a VAX/VMS microcomputer. The model was calibrated with TCE in precision-cut mouse liver slices *in vitro* (Byczkowski et al., 1995), and B6C3F1 mice treated with TCE *in vivo* (Byczkowski et al., 1997a). The module for TBARS production in precision-cut mouse liver slices, confirmed with experimental data, was published elsewhere by Byczkowski et al. (1996). The module for ethane exhalation, calibrated with experimental data from B6C3F1 mice, was presented previously by Byczkowski and Seckel (1996).

The PBPK Module for TCE in B6C3F1 mice was built as a conventional PBPK model for volatile chemicals (Gargas et al., 1989), confirmed with data from the literature, and was presented elsewhere by Das et al. (1994).

The BBPD module for cellular target inhibition by one-hit or multi-hit mechanisms, confirmed with data from the literature, was based on either deterministic or stochastic equation described previously by Byczkowski and Flemming (1996). Details of modeling approach, modeling parameters and computer codes are available in a recent technical report (Byczkowski et al., 1997a).

Basic framework

The modular structure of the interlinked PBPD model was designed in such a way that the output of each preceding module, representing a function of the process parameters, was directed as an input into the next module. At first, in the PBPK module the internal delivered dose of TCE has been described as a function of exposure with an appropriate pharmacokinetic (PBPK) model. Then, the local concentration of free radicals, generated by TCE, has been described as a function of the local dose of the chemical with a "square root algorithm." Next, using the available mechanistic information, oxidative stress and lipid peroxidation have been described as functions of free radical concentration over time. Finally, the cellular effects were described quantitatively by a biologically based dose-response (BBDR) module as continuous or discrete phenomena, depending on the magnitude of oxidative stress. Two cases of the dependence were considered: deterministic and stochastic, as described below.

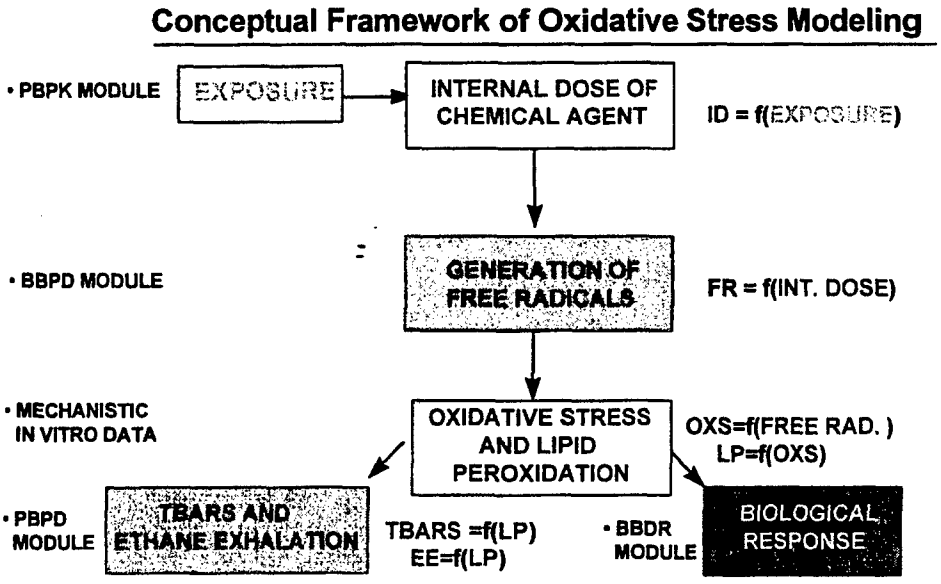

Figure 6.1-1. **A conceptual framework for quantitative modeling and dose-response characterization of the chemically induced oxidative stress. ID - internal dose (delivered); FR - free radicals; OXS - oxidative stress; LP - lipid peroxidation; TBARS - thiobarbituric acid reactive substance; EE - ethane exhalation.**

Deterministic algorithm

FRAD + CELLULAR TARGET → INACTIVE TARGET
assumptions: uniform sensitivity of CELLULAR TARGETS to FRAD

$In = I_0 \times \exp(-k_d \times FRAD \times t_p)$

where: I_0 is the initial concentration of active cellular targets, assumed to be 100% (I_0 = 1.0 [%/100]); k_d is the rate constant of cellular target inactivation by free radicals [100%/mM/h]; In is a fraction of remaining active cellular targets, relative to the amount before inhibition [ratio]; t_p is time of preincubation with free radicals [h]; FRAD is the concentration of free radicals [mM].

Stochastic algorithm

FRAD + MULTIPLE TARGETS → RANGE OF INHIBITORY RESPONSES

assumption: normal distribution of the INHIBITORY RESPONSES

$$PROB = 1 - Ft \times \int 1/\sqrt{2\pi} \times \exp(-z^2/2) \times dz$$

where: x = (DOS - M)/SD; PROB is probability of cellular targets to remain active, relative to the amount before inhibition [ratio] and may be expressed as a percentage of uninhibited (control) activity; Ft is a fraction [ratio] of incubation time needed to reach the maximum effect ($Ft = t_p/T_{ME}$), DOS is array of free radical concentration values [mM], between maximum "no effect" and minimum "100% effect" doses; M is mean of the cumulative Gaussian

distribution of free radical concentration values [mM]; SD is standard deviation of the cumulative Gaussian distribution of free radical concentration values [mM]; z is a variable of integration.

RESULTS AND DISCUSSION

Because biological responses of tissues and organs are mechanistically linked with local concentrations of active forms of a xenobiotic, the internal dose of chemical that reaches a particular physiological compartment must be used for any meaningful risk characterization. However, without understanding "what the particular dose of a chemical can do to the organism" the whole process of risk characterization could be useless to a risk manager.

In addition to the question about the direct biological effect of the delivered concentration of an activated (free radical) form of the pro-oxidant chemical, other important questions to answer are: "How is the target organ protected against free radical insult?"; "How fast are normal (physiological) processes of autooxidation?"; and "How fast do the activating enzymes (CYP) degrade?" We tried to address these questions in experiments with known drastic pro-oxidants: CCl_4 and $BrCCl_3$ (Byczkowski et al., 1997b and 1997c).

For quantitative characterization and modeling of the dose-response for a weak pro-oxidant chemical such as TCE, it was necessary to assume that its mode of action is qualitatively the same as that of drastic pro-oxidants. These considerations, based on the available literature (Larson and Bull, 1992; Cojocel et al., 1989) and experiments conducted in our laboratory (Das et al., 1994, Byczkowski and Seckel, 1996), led to the development, calibration, and partial verification of the interlinked PBPD model for TCE induced oxidative stress (Figure 6.1-1). We assumed that TCE is bioactivated by CYP (mainly in the liver) to metabolites producing free radicals and initiating lipid peroxidation (Larson and Bull, 1992; Cojocel et al., 1989). Our EPR spin-trapping study of mouse liver treated *in vitro* with TCE confirmed the increased production of free radicals (Steel-Goodwin et al., 1996). Therefore, the parameters of lipid peroxidation and the feasibility of square root algorithm, which links concentration of pro-oxidant with production of free radicals, were determined by our experiments with mouse liver slices treated *in vitro* with CCl_4 and $BrCCl_3$ and compared with those for TCE.

At the next step, the yield of ethane generation from lipid hydroperoxides was estimated (EF_{fe} = 0.001 [molar ratio]) by comparing the simulated molar amounts of ethane *in vivo* with TBARS *in vitro*, produced in response to the same total cumulated dose (area under the concentration curve, AUC) of TCE in the liver. Finally, the calibrated PBPD sub-model was verified with our data for ethane exhalation induced by three different doses of TCE (Figure 6.1-2). The calibrated algorithm and the *in vitro* estimated parameters were included in the PBPD model simulations of time- and dose-dependent effects of different doses of TCE on ethane exhalation in B6C3F1 mice *in vivo* (Figure 6.1-2). At this verification step, the parameters estimated during the *in vitro* and *in vivo* calibration with TCE were not further adjusted.

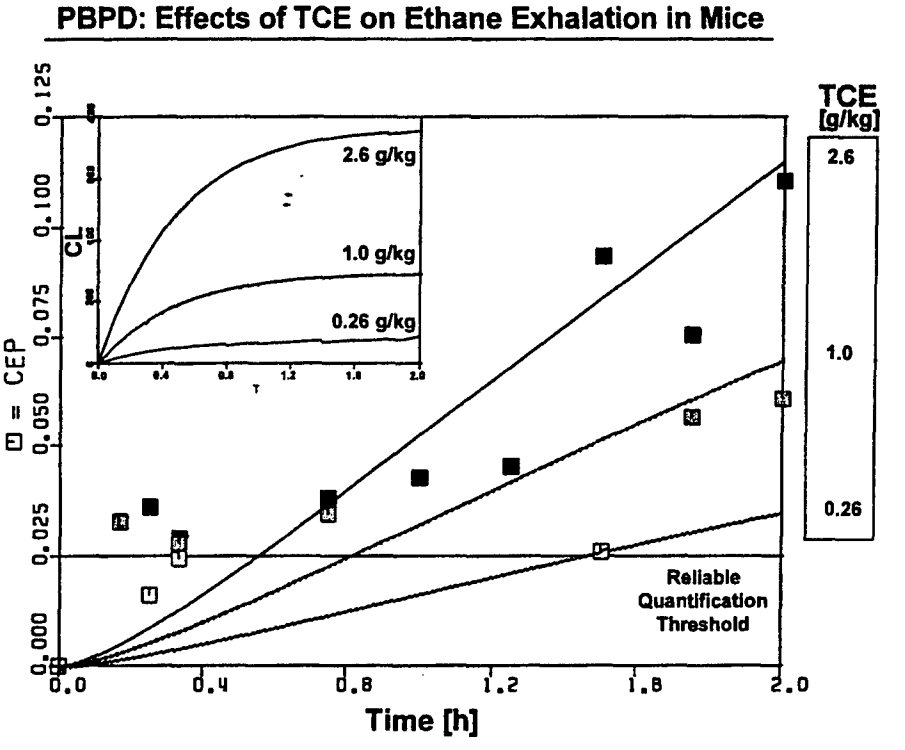

Figure 6.1-2. **The results of PBPD model simulations of our experimental data from mice treated i.p. with three different doses of TCE (Γ-0.26, -1.0, and O-2.6 g/kg) and exhaling ethane in a closed chamber (Byczkowski et al., 1996).**
CEP - concentration of ethane in a closed chamber [ppm]; Time - [h]. Symbols depict experimental data collected from the closed chamber (five animals per time-point) with the respective physiological ethane exhalation values (background) from the same group of five mice, measured before treatment, subtracted from the data points. Lines are the PBPD model simulations. The horizontal line depicts a reliable ethane quantification threshold by the method used (detectibility threshold + 2 S.D.). Inset shows PBPK sub-model simulations of local TCE concentrations in the liver. CL - local concentration [mg/kg liver]; T - time [h].

Deterministic Module

The deterministic BBDR module (Byczkowski and Flemming, 1996) was calibrated with experimental data of Vroegop et al. (1995) for inhibition of amino acid and glucose transporters in N 18 neuronal hybridoma cell line in culture, incubated with cumene hydroperoxide (Figure 6.1-3A) or hydrogen peroxide (Figure 6.1-3B), respectively, Heffetz et al. (1990) and Hecht and Zick (1992) for inhibition of protein tyrosine phosphatase (PTyrPase) in rat hepatoma cells in culture incubated with hydrogen peroxide (Figure 6.1-3C) or vanadate (Figure 6.1-3D), respectively. The rate constant for quenching of free radicals by all biological systems investigated (k_f) was estimated as 200.0 [1/μM/h] and was fixed during all simulations. Rate constants of free radical formation (k_i [1/μM/h]) were chemical-specific and rate constants of cellular target inhibition (k_d [1/μM/h]) were biological system-specific and

were optimized with SIMUSOLV® software. Initial concentrations of active targets were assumed to be 100% in uninhibited biological systems before incubation with pro-oxidant chemicals (I_0 = 1.0 [%/100]). Timing parameters [h] were dependent on the experimental setup. The final simulation parameters are listed in Table 6.1-1 (Byczkowski and Flemming, 1996).

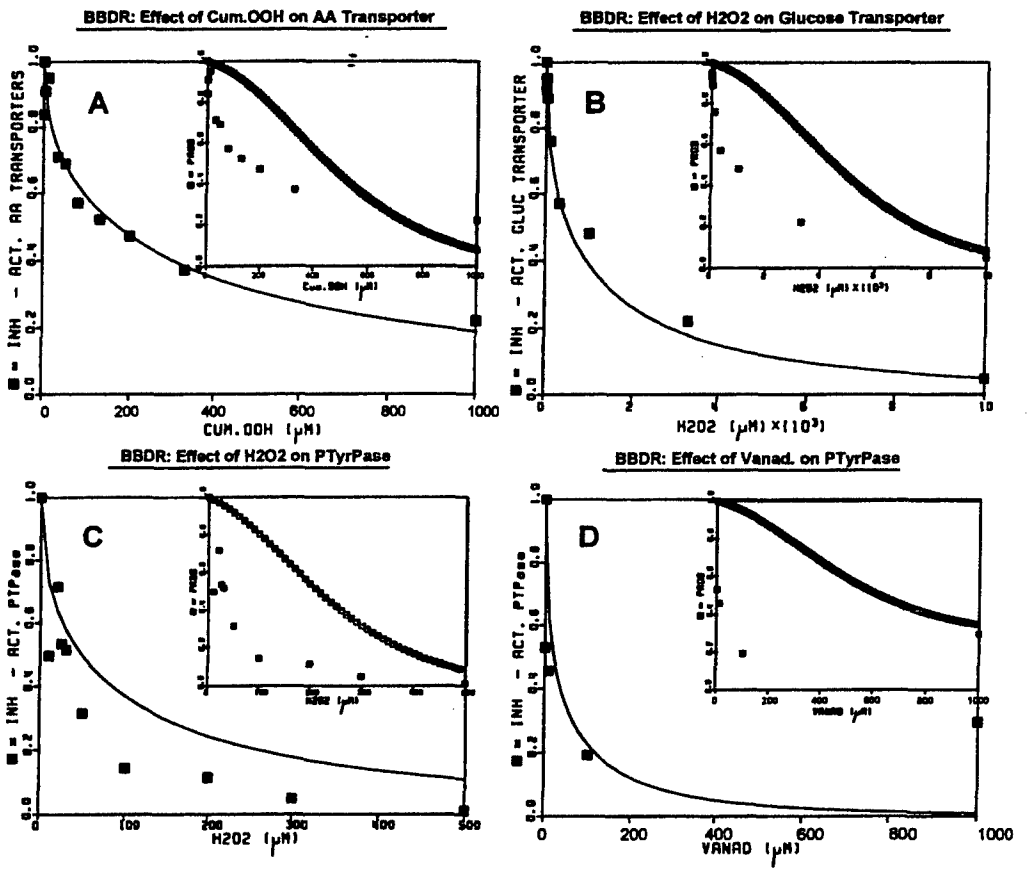

Figure 6.1-3. **Calibration of the deterministic BBDR module with *in vitro* data for inhibition of cellular targets by free radicals generated by pro-oxidant chemicals. Lines are computer-generated simulations by the deterministic BBDR module. Black squares depict data from the literature. Insets show, for comparison, simulations by the stochastic BBDR module (curves depicted by empty squares) with the same data (black squares). Optimized parameters are listed in Table 6.1-1.**

A. Effects of different concentrations of cumene hydroperoxide (Cum.OOH [μM]) on activity of amino acid (AA) transporter in N 18 neuronal hybridoma cell line in culture (INH [ratio]) after 1 h (*plus* 1 h preincubation with Cum.OOH; data points from Vroegop et al., 1995). B. Effects of different concentrations of hydrogen peroxide (H_2O_2 [μM]) on activity of glucose transporter (Gluc) in N 18 neuronal hybridoma cell line in culture (INH [ratio]), after 1 h (*plus* 1 h preincubation with H_2O_2; data points from Vroegop et al., 1995). C. Effects of different concentrations of hydrogen peroxide (H_2O_2 [μM]) on activity of protein tyrosine phosphatase (PTyrPase) in rat hepatoma cells in culture (INH [ratio]) after 5 min (*plus* 20 min preincubation with H_2O_2; data points from Heffetz et al., 1990). D. Effects of different concentrations of vanadate (Vanad [μM]) on activity of protein tyrosine phosphatase (PTyrPase) in rat hepatoma cells in culture (INH [ratio]), after 8 min (*plus* 0.5 h preincubation with vanadate; data from Hecht and Zick, 1992).

Stochastic Module

The stochastic BBDR module (Byczkowski and Flemming, 1996) was calibrated with experimental data of Vroegop et al. (1995) for inhibition of amino acid transporter and mitochondrial activity in N 18 neuronal hybridoma cell line in culture, incubated with 6-OH dopamine (Figures 6.1-4A and 6.1-4C) or hydrogen peroxide (Figures 6.1-4B and 6.1-4D), respectively. Optimized simulation parameters are listed in Table 6.1-1 (Byczkowski and Flemming, 1996).

TABLE 6.1-1. PARAMETERS FOR THE BIOLOGICALLY BASED PHARMACODYNAMIC MODEL DESCRIBING THE INHIBITION OF CELLULAR TARGETS BY CHEMICALLY GENERATED FREE RADICALS (after Byczkowski et al., 1997b).

Parameter	Description	Numerical value
	Rate constants of FR formation [1/μM/h]	
ki	from Cum.OOH	100.0[a]
ki	from 6OHD	200.0[a]
ki	from H_2O_2, vanadate and pervanadate	18.0[a,d]
ki	from TCE	900.0[b]
	Rate constant of FR quenching [1/μM/h]	
kt	fixed for all biological systems	200.0[c]
	Rate constants of cellular target inhibition [1/μM/h]	
kd	AA transporter	0.075[a]
kd	mitochondria	0.05[a]
kd	Gluc. transporter	0.1[a]
kd	PTyrPase	1.0[d]
	Initial concentration of active targets [%/100]	
I_0	assumed value for all biological systems	1.0

Abbreviations: Cum.OOH - cumyl hydroperoxide; 6-OHD - 6-hydroxy dopamine; TCE - trichloroethylene; FR - free radicals; AA - amino acids; Gluc. - glucose; PTyrPase - protein tyrosine phosphatase.

[a] fitted to Vroegop et al. (1995).

[b] fitted to Steel-Goodwin et al. (1995).

[c] estimated from Vroegop et al. (1995), Steel-Goodwin et al. (1995), Heffez et al. (1990), and Hecht and Zick (1992).

[d] fitted to Heffez et al. (1990) and Hecht and Zick (1992).

Predictive BBDR Model Simulations

Assuming that the secondary and tertiary free radicals generated by different pro-oxidant chemicals have the same effect on oxidant-sensitive enzymatic activities (e.g., on oxidation of -SH domain of the protein tyrosine phosphatase), it was possible to simulate the extent of enzyme inhibition by oxidative stress caused by TCE at the internal, local doses relevant to those attained during our i.p. experimental treatment of mice *in vivo* (Figure 6.1-2). Within the appropriate range of concentrations, the deterministic (Figure 6.1-5) and stochastic (Figure 6.1-6) predictive BBDR model simulations (unconfirmed experimentally with TCE) showed quite different dose-dependent characteristics.

Predictions of the remaining percentage of the enzymatic activity for oxidant-sensitive liver enzymes, showed greater impact on those inhibited specifically by TCE-derived free radicals (especially at low doses and during short exposure) than on those interacting non-specifically (compare Figures 6.1-5 and 6.1-6). However, the difference became negligible at a high TCE concentration (corresponding to the i.p dose of 2.6 g/kg) and during long exposure (2 h). Of course, these predictions should be verified experimentally by measuring the effect of TCE exposure on the enzymatic activity of a system that is likely to be inhibited specifically by oxidative stress (e.g., protein tyrosine phosphatase), and at least one system likely to be non-specific (e.g., mitochondria).

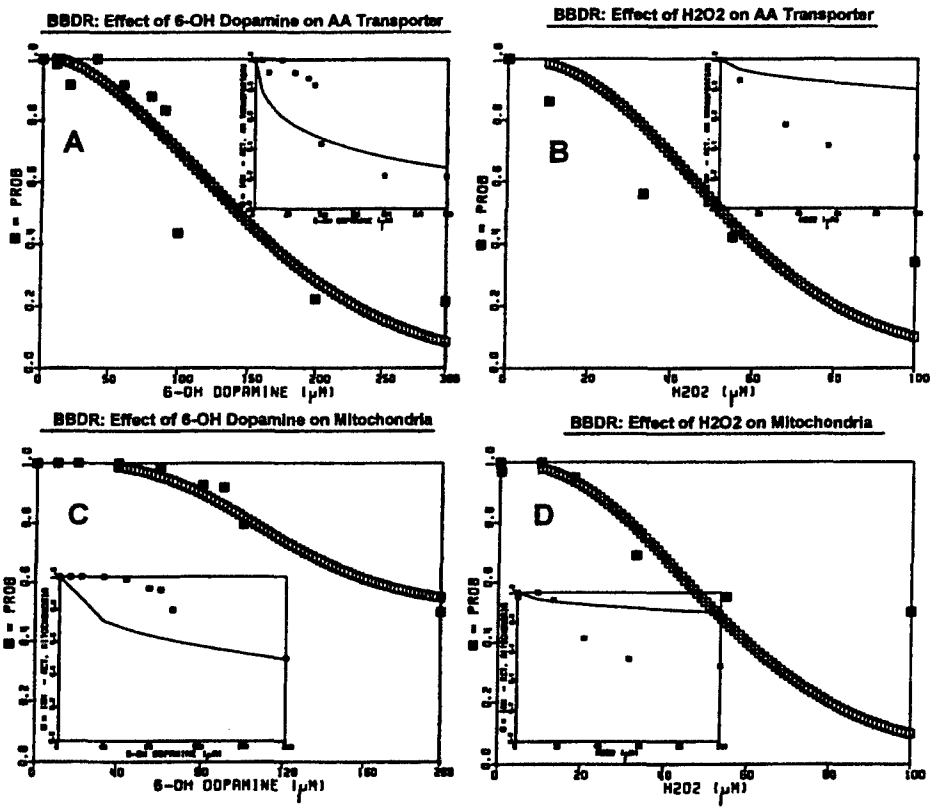

Figure 6.1-4. **Calibration of the stochastic BBDR module with *in vitro* data for inhibition of cellular targets by free radicals generated by pro-oxidant chemicals. Curves depicted by empty squares are computer-generated simulations by the stochastic BBDR module. Black squares depict data from the literature. Insets show, for comparison, simulations by the deterministic BBDR module (lines) with the same data (black squares). Optimized parameters are listed in Table 6.1-1.**

Effects of different concentrations of 6-hydroxy dopamine (6-OH Dopamine [μM]) on activity of amino acid (AA) transporter in N 18 neuronal hybridoma cell line in culture (PROB [ratio]), after 1 h (plus 1 h preincubation with 6-OH dopamine; data points from Vroegop et al., 1995). B. Effects of different concentrations of hydrogen peroxide (H_2O_2 [μM]) on activity of amino acid transporter (AA) in N 18 neuronal hybridoma cell line in culture (PROB [ratio]), after 1 h (plus 1 h preincubation with H_2O_2; data points from Vroegop et al., 1995). C. Effects of different concentrations of on activity of mitochondrial reduction of MTT (Mitochondria) in N 18 neuronal hybridoma cell line in culture (PROB [ratio]), after 1 h (plus 1 h preincubation with 6OHD; data points from Vroegop et al., 1995). D. Effects of different concentrations of hydrogen peroxide (H_2O_2 [μM]) on mitochondrial reduction of MTT (Mito) in N 18 neuronal hybridoma cell

line in culture (PROB [ratio]), after 1 h (plus 1 h preincubation with H_2O_2; data points from Vroegop et al., 1995).

Once validated, our BBDR model may be very useful for planing the doses and designing the treatment regime for future experiments. On the other hand, an analysis of the dose-response relationship with our model may be useful in establishing the mode of action (targeted *versus* random) for TCE and other pro-oxidant chemicals.

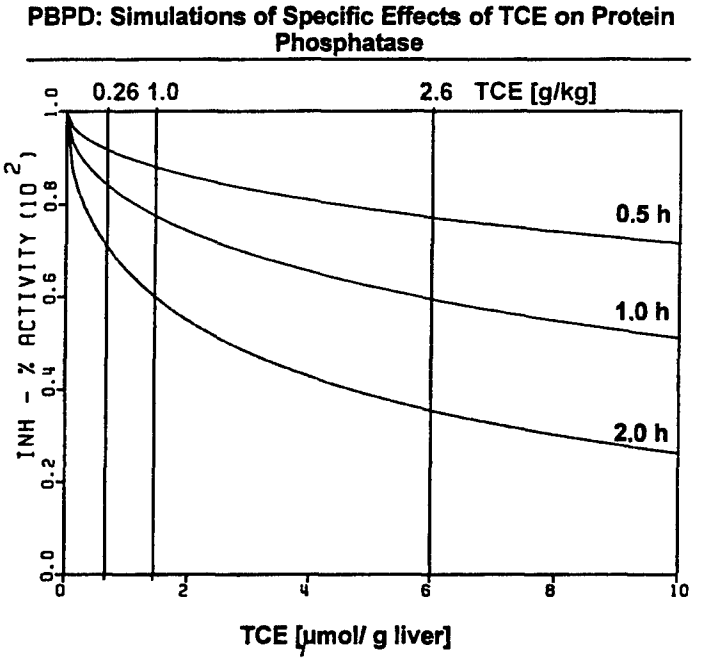

Figure 6.1-5. **The results of PBPD model deterministic simulations of expected inhibitions of cellular targets in the liver of mice after different times from beginning treatment with different doses of TCE (assumed k_d = 0.1 [100%/mM/h]; t_p = TSTOP). INH - remaining percentage of uninhibited activity x 10^2; TCE - local concentration of TCE in the liver [µmol/g].**

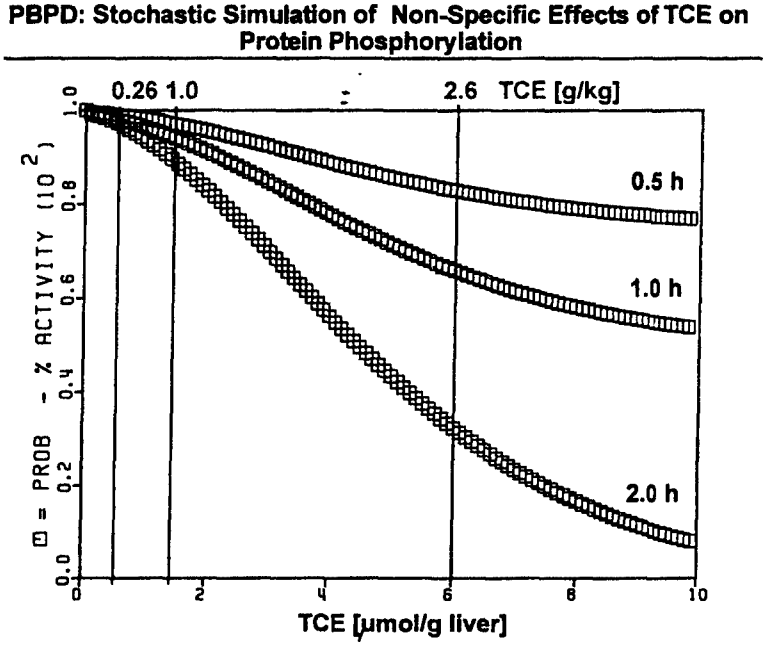

Figure 6.1-6. **The result of PBPD model stochastic simulation of expected inhibitions of cellular targets in the liver of mice after different times from treatment with different doses of TCE. PROB - percentage of uninhibited activity x 10^2; TCE - local concentration of TCE in the liver [µmol/g]; for other parameters, see Figure 6.1-5.**

The resultant computer-assisted simulation model described in this report may be allometrically scaled to reflect human physiology and metabolism, and may be included as a module in a predictive mechanistically based model (Figure 6.1-7) useful for risk characterization of pro-oxidant chemicals. This interlinked physiologically based pharmacodynamic model still should be validated with a discrete or continuously responding end-point (PATHOPHYSIOLOGY) measured as an ultimate biomarker in human subjects.

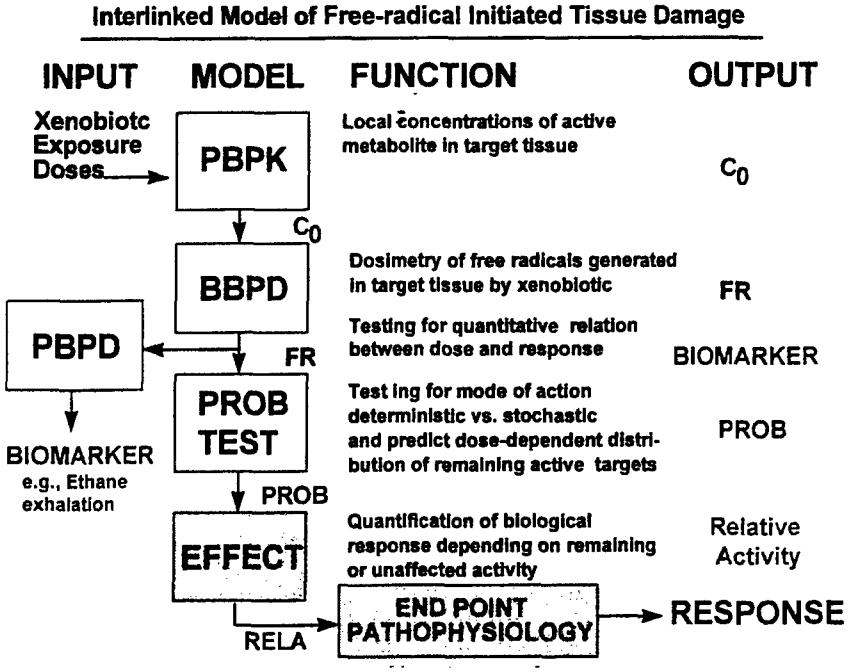

Figure 6.1-7. **For validation of the interlinked physiologically based pharmacodynamic model for pro-oxidant effects of trichloroethylene, a discrete or continuously responding end-point (PATHOPHYSIOLOGY) should be measured as an ultimate biomarker.**

CONCLUSION

The resultant PBPK/BBDR interlinked PBPD model may be used for pharmacodynamic description of oxidative stress in mice caused by TCE and potentially may be useful for its risk characterization.

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6.2 TOXICOKINETICS OF WATER SOLUBLE CHEMICALS IN THE ISOLATED PERFUSED RAT LIVER: TRICHLOROACETIC ACID

J.H. Toxopeus and J.M. Frazier[1]

ABSTRACT

The Isolated Perfused Rat Liver (IPRL) is a useful tool to study the role of the liver in the kinetics and metabolism of chemicals. One major advantage over liver homogenates, isolated hepatocytes, or liver slices is the potential to study mechanisms of biliary excretion. Trichloroacetic acid (TCA) has been used in this laboratory to develop a biologically based kinetic model for the IPRL for water soluble compounds. Studies performed with 25 μM or 250 μM TCA in perfusion medium resulted in a low, linear rate of excretion of TCA in bile. Enzyme leakage and bile production did not change in the presence of TCA, indicating that these TCA concentrations were not cytotoxic.

INTRODUCTION

Predicting the kinetics of xenobiotics in biological systems is important in the process of risk assessment. Biologically based kinetic (BBK) models have been developed for this purpose. So far most BBK models focused on lipid soluble compounds using partitioning coefficients to describe tissue uptake under perfusion limited conditions. These models do not apply for water soluble compounds such as metals and organic anions for which the cell membrane forms a barrier for uptake and their distribution is limited by diffusion rather than perfusion. The liver is a major controlling factor in determining systemic kinetics of many chemicals. The Isolated Perfused Rat Liver (IPRL) is a useful *in vitro* system to study the role of the liver in the kinetics and metabolism of chemicals. One major advantage over liver homogenates, isolated hepatocytes, or liver slices is the potential to study mechanisms of biliary excretion. An advantage of this system over *in vivo* experiments is the lack of interference of systemic factors and easy experimental manipulation of chemical concentration in the perfusion medium. A Biologically Based Kinetic (BBK) model for the IPRL for water soluble compounds is being developed.

The first chemical chosen to develop this BBK model was trichloroacetic acid (TCA). TCA is a water soluble compound that is virtually not metabolized by liver (Bruschi and Bull, 1993), simplifying the BBK model. This chemical is one of the major metabolites of trichloroethylene, a common and persistent environmental contaminant. The *in vivo* formation of TCA from trichloroethylene may contribute to the hepatocarcinogenic effects observed in rodents exposed to trichloroethylene (Henschler, 1977). TCA has been shown to induce lipid peroxidation and peroxisome proliferation (Reddy et al. 1982), events which sometimes are related to the induction of hepatic tumors. Exposure of the IPRL to TCA will provide information on rate of uptake by the

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liver and biliary excretion of this compound. In addition, adverse effects of this compound on the liver can be studied, e.g. increased enzyme leakage and changes in bile production.

IPRLs were exposed to 25 μM or 250 μM TCA. Over a period of two hours the concentration of TCA was monitored in perfusion medium and in bile. The TCA concentration in liver was determined at the end of the experiment. These results will be used in the further development and evaluation of a BBK model for the IPRL. This BBK model for the liver can then be integrated in a BBK model for the whole rat.

METHODS AND MATERIALS

Materials

TCA and taurocholate were purchased from Sigma Chemical Co. (St. Louis, MO). The albumin used was low-endotoxin bovine serum albumin (A 2934). Preparation of the perfusion buffer is described in SOP No. PD-97-009. The IPRL system is described in SOP No. PD-97-011. In short: Livers were isolated from male Fisher 344 rats weighing between 244-290 g. Perfusion medium consisted of 200 mL Krebs-Ringer buffer supplemented with 11 mM glucose and 4% BSA, pH 7.40, 37 C. Medium was oxygenated with 95% O_2/5% CO_2 in a gas exchange chamber using silastic tubing. The pH was regulated by adjusting the O_2:CO_2 ratio. The perfusion flow rate was 40 mL per minute to assure adequate oxygenation of the liver. To sustain bile flow, taurocholate was infused at a rate of 33 μmoles per hour.

After isolation, the liver was allowed to stabilize for one hour before exposing the IPRL to 25 μM or 250 μM TCA for two hours. The TCA concentration of 250 μM was based on the initial serum concentration in rats i.v. dosed with 10 mg TCA per kg. TCA was analyzed by GC after derivatization to methyl esters (SOP No. 4300-1041). The concentration of unbound TCA was determined using ultrafiltration (Centrifree Micropartition device, Amicon Inc.,Beverly, MA) and measuring TCA in the filtrate.

Liver viability was monitored using the following parameters: percentage enzyme leakage in perfusion medium (lactate dehydrogenase) and bile flow ($\mu L * min^{-1} * g\ liver^{-1}$).

RESULTS

The kinetics of TCA in the IPRL system following exposure to 250 μM TCA is illustrated in Figure 6.2-1. The total TCA concentration in perfusion medium decreased slightly in the first 30 min then remained constant (Figure 6.2-1A). The majority of the TCA in perfusion medium, 93%, was bound to albumin. The TCA concentration

in bile was 40 μM (Figure 6.2-1B), lower than the total TCA concentration in perfusion medium but comparable to the free concentration. The concentration in bile remained constant throughout the experiment. Excretion in bile was linear in time and 52 ± 6 nmoles of TCA were excreted by the end of the experiment (Figure 6.2-1C), equivalent to 0.1% of the total dose. The average concentration of TCA in the liver, 38 ± 12 μmoles/kg, was lower than the total TCA concentration in perfusion medium. The unbound concentration of TCA in the liver was 32 ± 8 μmoles/kg implying that all TCA in liver was free since this was not significantly different from the total liver concentration.

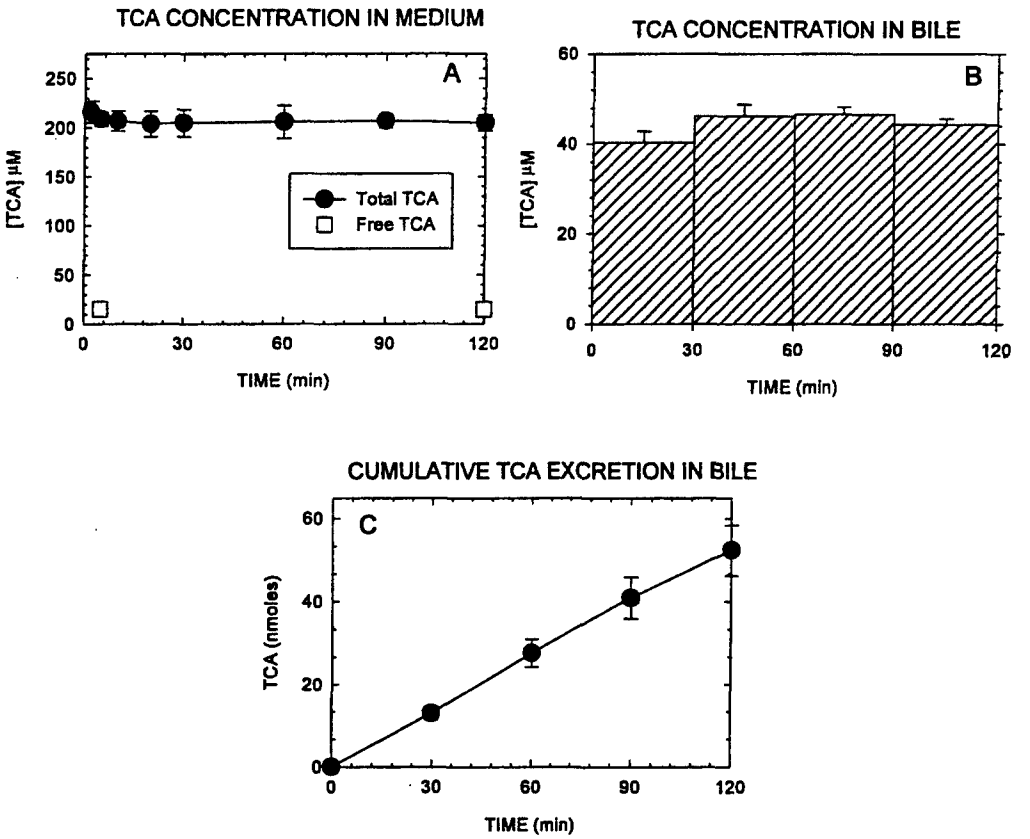

Figure 6.2-1. **(A) TCA concentration in perfusion medium.**
(B) TCA concentration in bile.
(C) Cumulative excretion of TCA in bile.

Rat livers were exposed for two hours in a recirculating IPRL system to 250 μM TCA. Results are expressed as the means ± SD of four separate experiments. Average liver weight was 9.0 ± 0.4 g.

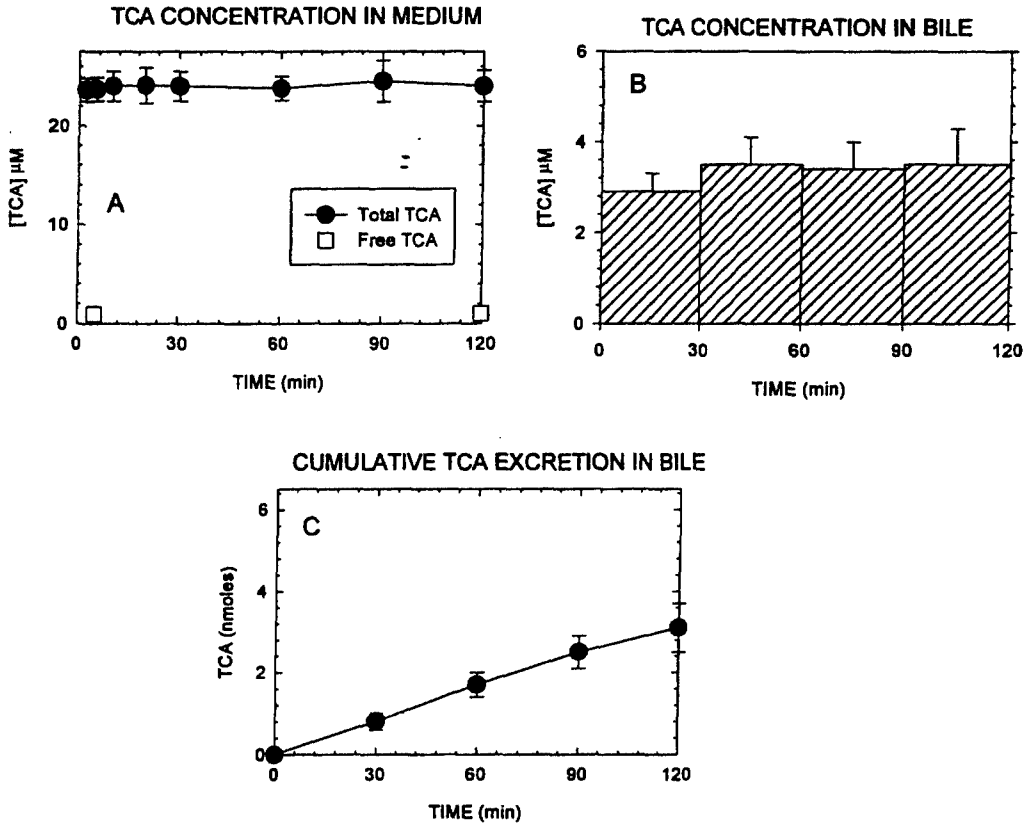

Figure 6.2-2. **(A) TCA concentration in perfusion medium.**
(B) TCA concentration in bile.
(C) Cumulative excretion of TCA in bile.

Rat livers were exposed for two hours in a recirculating system to 25 µM TCA. Results are expressed as the means ± SD of three separate experiments. Average liver weight was 10.1 ± 1.0 g.

Similar kinetic behavior was observed in the IPRL system at the 25 µM TCA exposure (Figure 6.2-2). The total TCA concentration in perfusion medium exhibited little change and 96% of TCA was bound to protein. The bile concentration was 4 µM and TCA excretion was linear in time with a total of 3.1 ± 0.6 nmoles being excreted during the experiment. The TCA concentration in the liver was 3.6 ± 0.9 µmoles/kg. The unbound concentration of TCA in the liver was 2.8 ± 0.6 µmoles/kg implying that all TCA in liver was free since this was not significantly different from the total liver concentration.

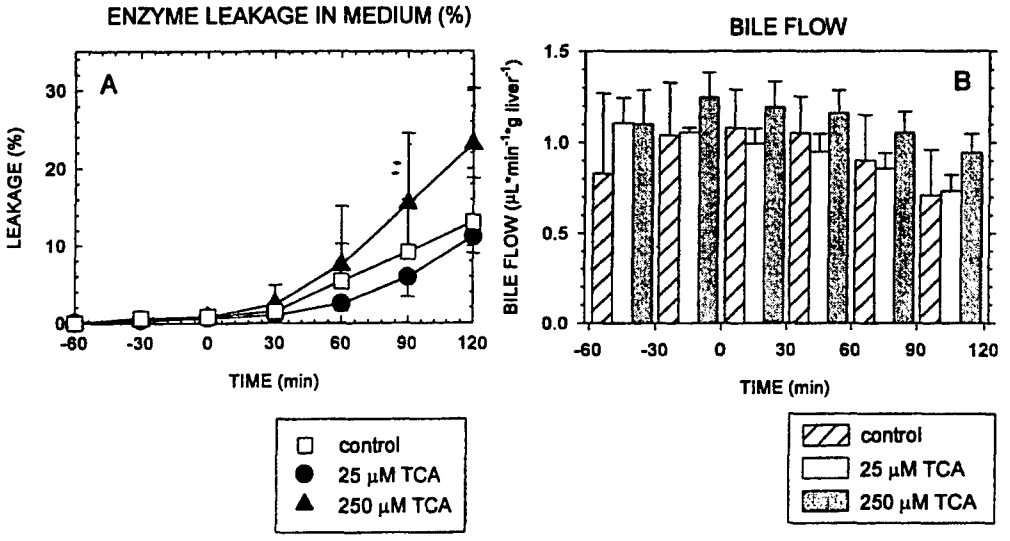

Figure 6.2-3. (A) Percentage LDH activity in perfusion medium. (B) Bile flow in the IPRL (µL * min^{-1} * g liver^{-1}).

Data are shown for control livers and livers exposed to 25 or 250 µM TCA.

The indicators for liver viability, enzyme leakag, and bile flow are shown in Figure 6.2-3. Enzyme leakage was not higher in TCA treated livers as compared to control livers (Figure 6.2-3A). Enzyme activity in perfusion medium was low during the first 90 min of exposure (t = – 60 to t = 30 min). After this period there was a gradual increase in enzyme activity in perfusion medium up to about 20% at the end of the experiment. Bile flow remained high in TCA treated livers (Figure 6.2-3B).

DISCUSSION

The results show that our IPRL system is suitable to study kinetics of chemicals during short studies (2 h). In addition to information on kinetics, potential toxic effects of chemicals can be monitored.

The results show that TCA is taken up by the liver and excreted in bile. The total amount excreted is only a very small portion of the total dose of TCA. This is similar to what was found when whole animals were dosed with TCA, namely that only 3% of the TCA was recovered in feces (Larson and Bull, 1992). Over 50% of the total TCA dose was excreted in urine in rats exposed via gavage at doses of 5, 20, and 100 mg TCA per kg. This pattern is consistent with low molecular weight chemicals (MW of TCA =163 Da). The reported threshold weight for organic anions to be preferentially excreted in bile is 325 Da for rats (Coleman, 1987). Organic anions with a lower molecular weight are preferentially excreted in the urine.

The kinetics of TCA were very similar at the two different doses used. The concentration in bile and liver was almost ten times lower when the liver was exposed to 25 μM as compared to 250 μM. The low uptake of TCA by the IPRL may result from preferential binding of TCA to albumin in perfusion medium relative to liver proteins. Almost 90% of TCA was bound to albumin in perfusion medium as compared to virtually no protein binding of TCA in liver homogenate. This differential binding behavior was confirmed in separate binding studies using untreated liver homogenates and untreated perfusion medium (results not shown).

One discrepancy between the *in vitro* experiments using the IPRL and results from *in vivo* experiments is that we did not find dichloroacetic acid (DCA) in our system. DCA has been found both in TCA treated rats and mice (Larson and Bull, 1992; Abbas et al., 1997). The formation of DCA from TCA was suggested to be mediated in the liver by P-450 via reductive dehalogenation. The analytical procedure used in these studies quantifies both TCA and DCA simultaneously, but no DCA was found in perfusion medium, liver or, bile (results not shown). However, it is possible that DCA may be further metabolized after formation keeping the DCA concentration below the detection limit. This is not unlikely since Larson and Bull (1992) found only about 1 μM DCA in blood plasma in rats dosed i.v. with 40 mg/kg TCA. Our dose of 250 μM TCA was based on serum concentrations of rats 30 min after they were dosed with 10 mg/kg TCA (personal communication, Dr. Kyung Yu). The detection limit for TCA and DCA is 2 μM. Our lower TCA dose makes it likely that the concentration of DCA will stay below the detection limit in our system. Furthermore, if DCA is formed, the rate of formation would be very low since there is minimal loss of TCA in our system. The fact that TCA is virtually un-metabolized by liver was actually one of the reasons this chemical was chosen for the development of the BBK model for the IPRL.

The fact that TCA was not cytotoxic in the IPRL system at these doses, as indicated by lack of effect on enzyme leakage and bile flow, agrees with results obtained using isolated hepatocytes. Bruschi and Bull (1993) observed that up to 1 mM TCA did not increase LDH leakage relative to control in isolated rat hepatocytes during four hours of exposure.

CONCLUSION

The study of TCA kinetics in the IPRL system provided important details about liver kinetics of TCA, indicating that differential protein binding in liver and perfusion medium may account for the low amounts of TCA taken up and excreted by the liver.

Acknowledgments

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6.3 ELECTROSPRAY ANALYSIS OF BIOLOGICAL SAMPLES FOR BROMOSULFOPHTHALEIN AND ITS GLUTATHIONE CONJUGATES

W.T. Brashear

ABSTRACT

Bromosulfophthalein is an organic dye molecule which is frequently used to test liver function. Bromosulfophthalein (BSP) is taken up by liver parenchymal cells, conjugated by glutathione-*S*-transferase, and excreted in the bile. Conjugation facilitates the transport of BSP from liver cells to bile, and glutathione conjugation is the rate limiting step in the transport of BSP from the blood to the bile. Much of the conjugated glutathione conjugated BSP is excreted in bile. BSP conjugation is useful for investigating the toxicological effects of chemicals. Specifically, it measures liver function by determining the ability of a chemical to interfere with the capacity of the liver to conjugate, and excrete BSP-glutathione conjugates into the bile.

Typically high-performance liquid chromatolography (HPLC) has been used for the analysis of bile for BSP and various BSP-glutathione conjugates. Spectrophotometric HPLC methods have limits of detection of approximately 0.1 μg/mL. This report describes an electrospray ionization mass spectrometry method for measuring BSP, BSP-glutathione, and BSP-cysteine conjugates in bile from perfused rat liver and perfusion media. Electrospray ionization mass spectrometry can simultaneously analyze several different conjugates at levels of sensitivity below 0.1 μg/mL.

INTRODUCTION

Bromosulfophthalein (BSP) is a brominated derivative of phenolsulfophthalein. Since BSP is excreted almost entirely by the liver, the rate of removal of BSP from blood has been used as a liver function test (Tietz, 1982). Removal of BSP is impaired by hepatocellular damage, and the bromosulfophthalein test is a sensitive index of hepatic dysfunction (Combes, 1959). However, the excretion of BSP can also be affected by hepatic blood flow, blood levels of BSP, and the condition of the bile duct. Significant retention of BSP may occur in conditions such as jaundice, intrahepatic disorders, and posthepatic disorders.

BSP is metabolized by glutathione-*S*-transferase to bromosulfophthalein monoglutathione conjugate (BSP-mGSH) (Wilco Snel et al., 1993). BSP is removed from blood and transported into liver cells where it is conjugated with glutathione and excreted into bile (Van't Klooster et al., 1988). Glutathione appears to be the only conjugate of BSP (Combes, 1959; Wilco Snel et al., 1993). Once inside the bile canaliculi, BSP-mGSH may be hydrolyzed to BSP-monocysteinylglycine and other conjugated metabolites. Among the conjugated metabolites found in bile are BSP-monoglutathione (BSP-mGSH), BSP-diglutathione (BSP-dGSH), BSP-monocysteine, (BSP-mCys), BSP-dicysteine (BSP-dCys), and BSP-monocysteinylglycine (BSP-mCys-Gly) (Sano et al., 1992). In male rats the

principle conjugate is BSP-mGSH (76%), which is followed by free BSP (14%), and BSP-mCys (4%) (Sano et al., 1992). The BSP monoconjugates have two positional isomers. The α-position, shown in Figure 6.3-1, is the predominant site of substitution. Isolated systems such as hepatocytes (Van't Klooster et al., 1988) and perfused liver (Wilco Snel et al., 1993) may be used to investigate the toxic effects of chemical compounds. The effect of chemical compounds on the transport and conjugation of BSP can provide information on the capacity of the compound to impair liver function.

The analysis of BSP and its conjugates from perfused liver and bile has been done via high-performance liquid chromatography (HPLC) (Wilco Snel et al., 1993). Many of these HPLC assays utilize the absorbance of BSP at 578 nm in alkaline pH (Van't Klooster et al., 1988, Sano et al., 1992). At pH values above the pKa of 8.8, BSP becomes a strongly absorbing chromophore. This is shown in Figure 6.3-1. The highly colored chromophore allows HPLC assays with spectrophotometric detection to have limits of detection as low as 80 ng/mL (Van't Klooster et al., 1988; Sano et al., 1992). Electrospray mass spectrometry for the analysis of bile and perfusion media for BSP, and its conjugated metabolites uses a completely different approach. The fact that BSP and its conjugated metabolites are negatively charged at physiological pH (see Figure 6.3-1.) permits them to be detected via negative ion electrospray mass spectrometry. Electrospray can detect BSP and different conjugates without chromatographic separation by quickly scanning different mass units. Tandem electrospray ionization mass spectrometry (ESI/MS/MS) with selected reaction monitoring can detect BSP and its metabolites at levels as low as 1 ng/mL.

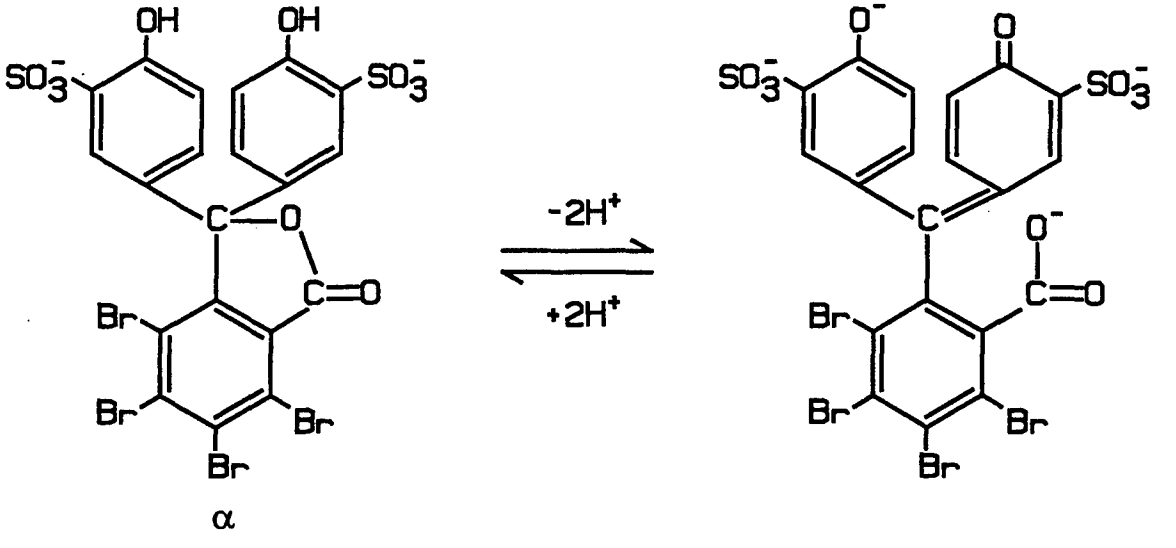

Figure 6.3-1. BSP molecule with α-bromine atom indicated. BSP at neutral pH exists as colorless anion (left). At pH values above the pKa (pH > 8.8) BSP loses two hydrogen ions to become a highly colored chromophore at 578 nm(right).

MATERIAL & METHODS

Chemicals & Equipment

BSP, L-cysteine, and acetonitrile were obtained from Aldrich Chemical Company (Milwaukee, WI). Glutathione and triethylamine were obtained from Sigma Chemical Company (St. Louis, MO). Methanol and anhydrous acetone were obtained from Fisher Scientific (Fairlawn, NJ). A Thermolyne model 16700 vortex mixer was used to vortex and incubate samples (Dubuque, IA). Samples were analyzed on a Finnigan TSQ 700 mass spectrometer equipped with a Finnigan electrospray interface (ThermoQuest Corp., Riviera Beach, FL). High performance liquid chromatography was done using a Hewlett-Packard 1050 solvent delivery system, a Hewlett-Packard 1050 autosampler, and a Hewlett-Packard 1050 UV monitor (Hewlett-Packard, Avondale, PA). Separation was done on a Supelcosil, 4.6mm x 25cm C-18 analytical column obtained from Supleco Chromatography Products (Bellefonte, PA).

Assay Procedure

Synthesis of Standards

Conjugates of BSP were made by a modification of the method of Whelan (Whelan et al., 1970). Reduced glutathione (736 mg) was added to a solution of BSP (40 mL at 50 mg/mL). The mixture was made basic to a pH of 10 by adding triethylamine (TEA). The reaction mixture was incubated for 2 h at 37 °C. After the incubation, 105 mL of anhydrous acetone was added, and the solution transferred to conical centrifuge tubes. The solution was centrifuged at 2000xg for 10 min, and the precipitate discarded. The supernatant was decanted to a 400 mL beaker and 4.5 volumes of anhydrous acetone were added for each 3.5 volumes of supernatant. This gave a gel-like precipitate consisting mainly of BSP-glutathione. The solution was centrifuged again as previously described, and the supernatant discarded. The resultant material was further purified by HPLC. The same procedure was used to synthesize the BSP conjugates of L-cysteine.

Bile samples were obtained from isolated perfused Fischer 344 rat liver. The perfused rat liver was dosed with TCA or DCA. Bile samples were collected before dosing, and at times of 0, 30, 60, and 90 min. The bile samples were diluted 1:50,000 prior to analysis. The Krebs Ringer bicarbonate perfusion media was also analyzed for BSP and its conjugated metabolites. Media was diluted 1:1000 prior to analysis.

Electrospray Mass Spectrometry

Bile samples and perfusion media samples were analyzed by electrospray mass spectrometry by simply diluting the samples with acetonitrile and water (80/20, v/v). The electrospray ionization tandem mass spectrometry (ESI/MS/MS) was performed in the negative ion mode. The ESI inlet was equipped with a 10 μL injection loop,

the flow rate was 100 μL/min, and the inlet capillary was held at 200 °C. The nitrogen sheath gas for the ESI interface was set at 35 psi, and no auxiliary gas flow was used. The electrospray voltage was set at 5 kV and current was 1 μA. Argon was used as the collision gas and the collision cell was operated at 1.5 millitorr, with an offset of 15 eV.

BSP was detected via selected reaction monitoring (SRM) in the neutral loss (nl) mode. Conjugates of BSP were also analyzed using SRM. Among them were BSP-mGSH, BSP-dGSH, BSP-mCys, and BSP-dCys. The mass settings used by the quads for the SRM analysis are shown in Table 6.3-1. The reactions which BSP, BSP-mGSH, and BSP-mCys undergo via SRM are shown in Figure 6.3-2. BSP, as shown in the top reaction of Figure 6.3-2, is a doubly charged anion with a m/z value of 396. Quad 1 is set at 396 allowing BSP to pass and enter the collision chamber, Quad 2. Here it collides with argon gas at 1.5 millitorr and looses CO_2. The daughter ion of this reaction has an m/z value 22 units lower than the parent ion, and Quad 3 is set to m/z 374 allowing it to pass to the detector. The SRM reactions of BSP-mGSH and BSP-mCys are shown in the second and third reactions of Figure 6.3-2, respectively. These were analyzed in the same manner as BSP. The only difference was the mass settings for Quad 1 and Quad 3. The scan time for the SRM was 0.14 s for each ion. Peak areas were manually integrated using the Finnigan CHRO program supplied with their proprietary ICIS software. First and second order polynomials, without weighting, were used for standard curves of BSP and its conjugates.

TABLE 6.3-1. SRM CONDITIONS

	Quad 1	Quad 2	Quad 3
BSP	396	22	374
BSP-Mgsh	509	159	350
BSP-dGSH	415	-71	486
BSP-mCys	415	44	371
BSP-dCys	436	87	349

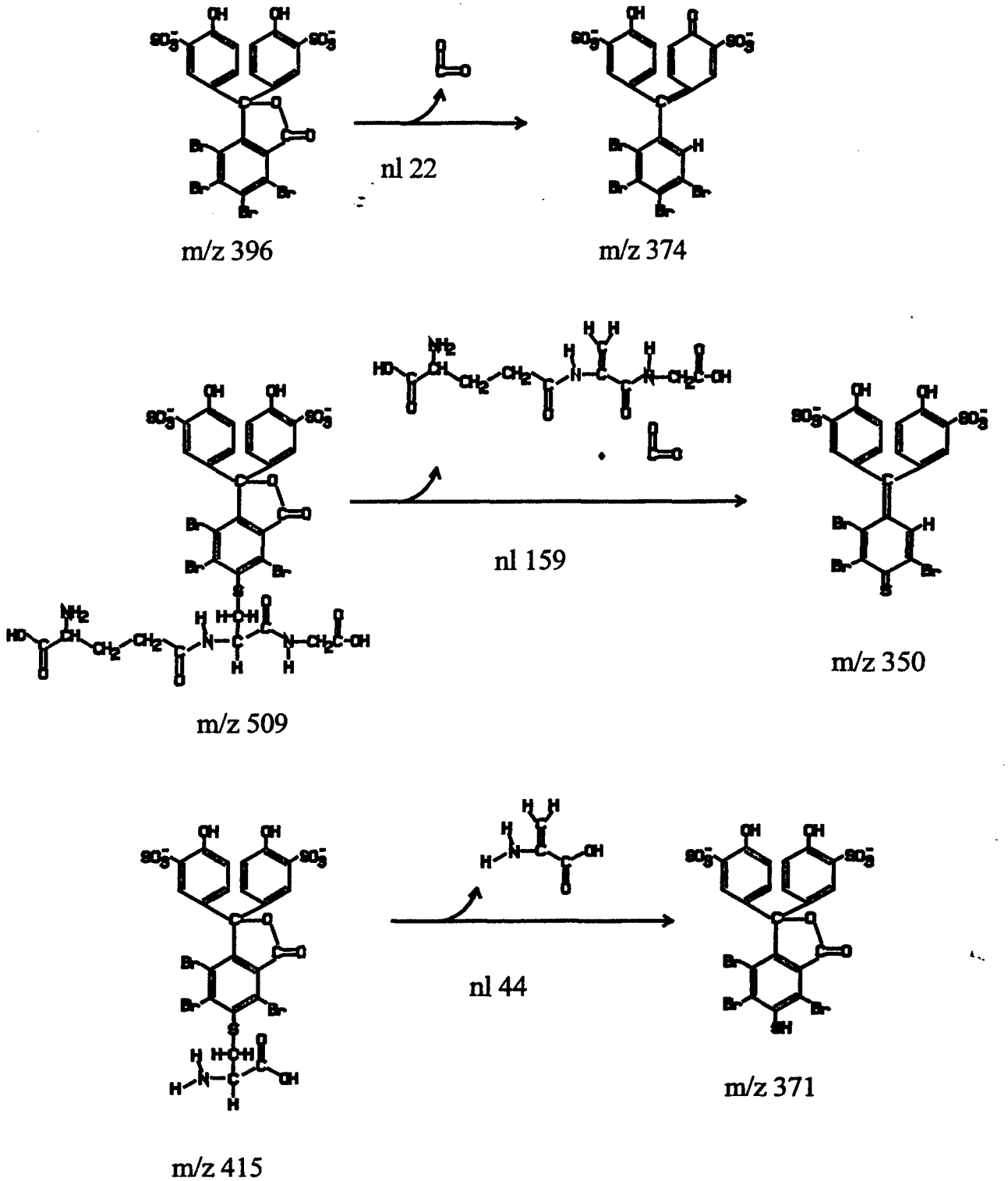

Figure 6.3-2. **SRM reactions. Top reaction, BSP (m/z 396) looses CO_2, a neutral loss (nl) of 22, to yield a doubly charged anion at m/z 374. Middle reaction, BSP-mGSH (m/z 509) undergoes a neutral loss of 159 to yield a doubly charged anion at m/z 350. Bottom reaction, BSP-mCys (m/z 415) undergoes a neutral loss of 44 to yield a doubly charged anion at m/z 371.**

RESULTS AND DISCUSSION

BSP, BSP-mGSH, and BSP-dGSH were found to give a concentration-dependent response when analyzed by electrospray mass spectrometry and SRM. This was found to be true for bile samples diluted 1:50,000 with acetonitrile and water (80/20, v/v) and perfusion media samples diluted 1:2000. Standard curves for BSP and BSP-mGSH in bile are shown in Figure 6.3-3.

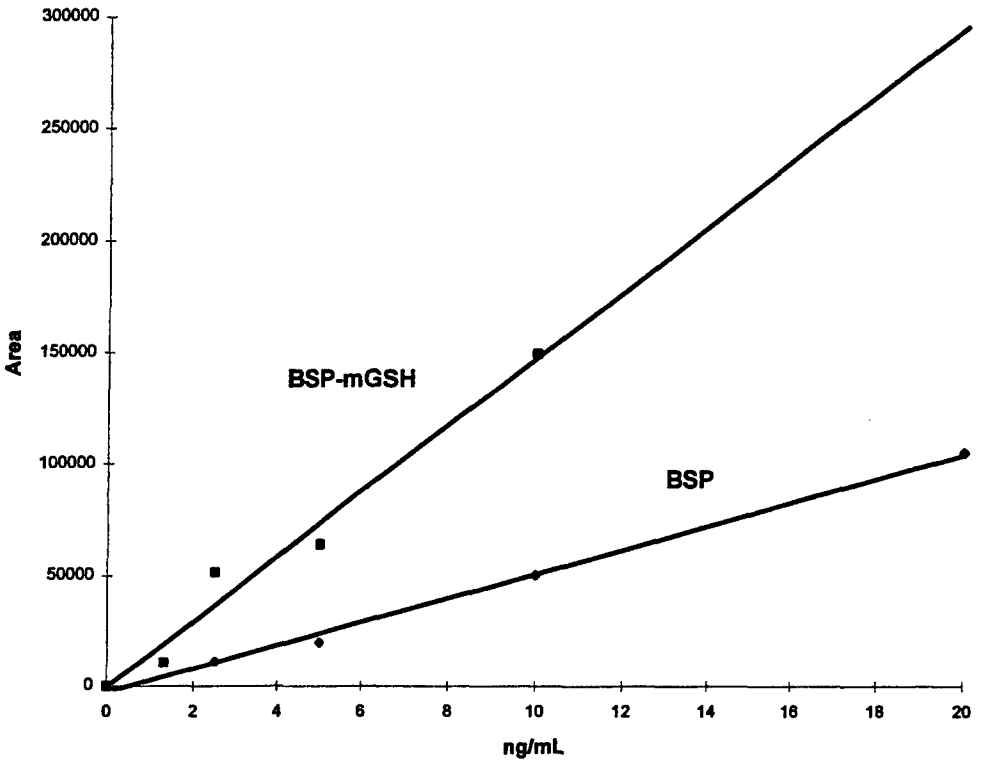

Figure 6.3-3. Standard curves for BSP and BSP-mGSH in bile. Area counts are plotted against concentration in ng/mL. BSP was plotted at concentrations of 2.5 to 20 ng/mL, and BSP-mGSH was plotted at 1.3 to 10 ng/mL.

Linear first order equations were obtained for BSP and BSP-mGSH in bile. The regression line equations for BSP and BSP-mGSH were: $y=5329x-2779$ ($r^2=0.996$) and $y=14756x-221$ ($r^2=0.973$), respectively.

Precision data for the analysis of BSP and BSP-mGSH are shown in Table 6.3-2. Since this is an ongoing study the data sets have a small number of replicates. Bile samples were diluted 1:50,000 and perfusion media samples were diluted 1:2000.

TABLE 6.3-2. BSP and BSP-mGSH Precision Data

BSP and BSP-mGSH in Bile				
	BSP		BSP-mGSH	
µg/mL	250	500	125	250
n	4	4	4	4
Mean	274	588	136	221
Accuracy*	9.5%	17.5%	9.0%	11.5%
SD	50	39	43	50
CV	18%	7%	31%	22%

BSP and BSP-mGSH in Perfusion Media				
	BSP		BSP-mGSH	
µg/mL	10	20	5	10
n	6	6	6	6
Mean	10.2	20.6	4.7	9.0
Accuracy*	1.7%	2.8%	6.0%	9.7%
SD	3.0	6.1	0.8	1.8
CV	30%	30%	18%	20%

*** Accuracy computed as percentage difference between the obtained and expected values, divided by the expected value.**

The precision data tended to be better for BSP and BSP-mGSH in perfusion media compared to bile. This may be due to the fact that perfusion media is a synthetic matrix. Bile, being a natural biological matrix, may contain macromolecules which can more tightly bind BSP and BSP-mGSH. This binding could hinder the desolvation of BSP and BSP-mGSH in the electrospray source. Incomplete desolvation will have a significant impact on the detector response of a molecule being analyzed by electrospray mass spectrometry. This is due to the fact that a molecule must undergo desolvation in the electrospray source before it can enter the analyzer section of the mass spectrometer.

BSP and the BSP-mGSH conjugate were found in bile samples from isolated perfused rat liver. BSP was present at levels of about 1000 µg/mL, and BSP-mGSH was present at levels of about 250 µg/mL. The concentrations were lower in perfusion media. Here, BSP was present at about 10 µg/mL, and BSP-mGSH was not found. Other conjugates of BSP such as the cysteine conjugates or the diglutathione conjugates were not detected in either bile or perfusion media. Currently this analysis is being used for an ongoing study on isolated perfused rat liver. The isolated perfused rat liver will be treated with different concentrations of TCA and DCA. One objective is to investigate their effect on the ability of the liver to metabolize BSP.

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6.4 INFLUENCE OF PROTEIN BINDING ON THE KINETICS OF CHEMICALS IN BIOLOGICAL SYSTEMS: EFFECT OF ALBUMIN ON THE KINETICS OF BROMOSULFOPHTALEIN IN THE ISOLATED PERFUSED RAT LIVER

J.H. Toxopeus and J.M. Frazier[1]

ABSTRACT

The isolated perfused rat liver (IPRL) is a useful *in vitro* system to study kinetics of xenobiotics. The relative contribution of 1) the rate of dissociation of Bromosulfophtalein (BSP) from bovine serum albumin and 2) the intrinsic liver uptake to the overall rate of BSP elimination by the liver was studied using the IPRL. The IPRL was exposed to BSP in recirculating perfusion medium containing 0, 1, and 4% albumin (w/v). The initial BSP concentration was 20 μM, and the duration of the experiment was 150 min. As expected, BSP disappeared faster from perfusion medium when the concentration of albumin was lower, reflecting a greater concentration of free BSP. The increased rate of BSP extraction from the perfusion medium correlated with an increased biliary excretion rate of total BSP (unconjugated and conjugated). The kinetic data were analyzed using a biologically based kinetic (BBK) model developed for the IPRL and published BSP-albumin binding rate constants. The analysis indicates that both the rate of dissociation of BSP from albumin and the intrinsic liver uptake contribute to the overall rate of BSP elimination.

INTRODUCTION

Bromosulfophtalein (BSP) has been used in our laboratory to study the functional integrity of the IPRL. This compound is routinely used to evaluate liver function, i.e., the rate of BSP disappearance from blood is determined as an indicator of liver functionality (Tietz, 1982). There is a broad selection of literature dealing with BSP metabolism and protein binding. The molecular weight of BSP is 838 Da, well above the threshold weight for biliary excretion (325 Da, Coleman). The molecular weight of BSP is increased to above 1000 Da upon formation of mono- and di-glutathione conjugates. Both parent compound and conjugates are excreted in bile. The concentration of BSP and its conjugates is significantly higher in bile than in the sinusoids, suggesting active transport into bile. Important factors contributing to the kinetic behavior of compounds are metabolism, membrane transport, and protein binding.

In general it is assumed that the uptake rate of chemicals is determined by the concentration of free compound. The association and dissociation of compounds to protein is thought to occur fast enough to obtain equilibrium between free and bound ligand in the sinusoids. BSP binds to protein and literature values for binding of BSP to

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albumin are available (Baker and Bradley 1966; Pfaff et al 1975, Weisiger et al., 1984, Zhao et al., 1993). The IPRL can be easily used to study factors important in BSP kinetics. Advantages over *in vivo* studies is the lack of interference from systemic factors and the easy experimental manipulation of conditions in the perfusion medium. Advantages over other *in vitro* systems are the intact liver architecture and biliary excretion in the IPRL system.

To study the role of protein binding in BSP kinetics three different albumin concentrations in perfusion medium were used: 0, 1, and 4% (w/v) (0 μM, 143 μM, and 570 μM, respectively). The initial BSP concentration used in all experiments was 20 μM. By applying a BBK model for the IPRL (Foy, 1997) to analyze the kinetic data, the role of protein binding in BSP kinetics was studied. The results presented here indicate that the rate of uptake may be limited by the rate of dissociation of BSP from albumin.

MATERIALS AND METHODS

Materials

BSP, albumin, and taurocholate were purchased from Sigma Chemical Co. (St Louis, MO). Albumin used was low-endotoxin bovine serum albumin (A 2934).

Animals

Male Fischer rats weighing between 230-300 g were used for liver isolations. Rats had free access to food and water purified by reversed osmosis. Preparation of the Krebs-Ringer buffer used is described in SOP No. PD-97-009. Experimental procedures for the preparation of the IPRL are described in SOP No. PD-97-011. Perfusion medium consisted of 200 mL Krebs-Ringer buffer supplemented with 11 mM glucose, pH 7.40, 37 C. Medium was oxygenated with 95% O_2/5% CO_2 in a gas exchange chamber using silastic tubing. The pH was regulated by adjusting the O_2:CO_2 ratio. The flow rate was 40 mL per min to assure adequate oxygenation of the liver. To sustain bile flow, taurocholate was infused at a rate of 33 μmoles per hour. After isolation, the liver was allowed to stabilize for 30 min before dosing the IPRL system with 4 μmoles of BSP giving a final BSP concentration of 20 μM. Perfusions in the presence of BSP were continued for 150 min. Perfusion medium contained either 0, 1, or 4% (w/v) albumin.

Liver viability was monitored using enzyme leakage (lactate dehydrogenase), bile flow (μL * min^{-1} * g $liver^{-1}$), and histopathology.

To analyze BSP in perfusion medium, 0.5 mL 1N NaOH was mixed with 0.5 mL perfusion medium. This mix was transferred to cuvettes and absorbance at 580 nm was read. To quantify BSP in bile, 1 mL perfusion medium

was mixed with 1 mL 1N NaOH, and 10 µL bile was added. The mixture was transferred into cuvettes and the absorbance was read at 580 nm. Bile collected before BSP exposure was used as blank. To calculate the concentration of BSP in perfusion medium and bile, a standard curve of BSP absorbance at 580 nm was generated in SIGMA plot (Jandel Scientific).

Model Implementation

The BBK model was coded on a PC using Advanced Computing Simulation Language (ACSL level 11, MGA Associates, Concord, MA), a numerical integration package. For details, see Foy, (1997).

RESULTS

The kinetics of BSP in the perfusion medium is illustrated in Figure 6.4-1. In the absence of albumin almost all BSP was extracted from the perfusion medium by the IPRL within the first 30 min of exposure. The disappearance of BSP from perfusion medium was slower in the presence of 1% albumin but almost complete by the end of the experiment. The liver extracted only 80% of the BSP from perfusion medium in the presence of 4% albumin during the 150 min exposure.

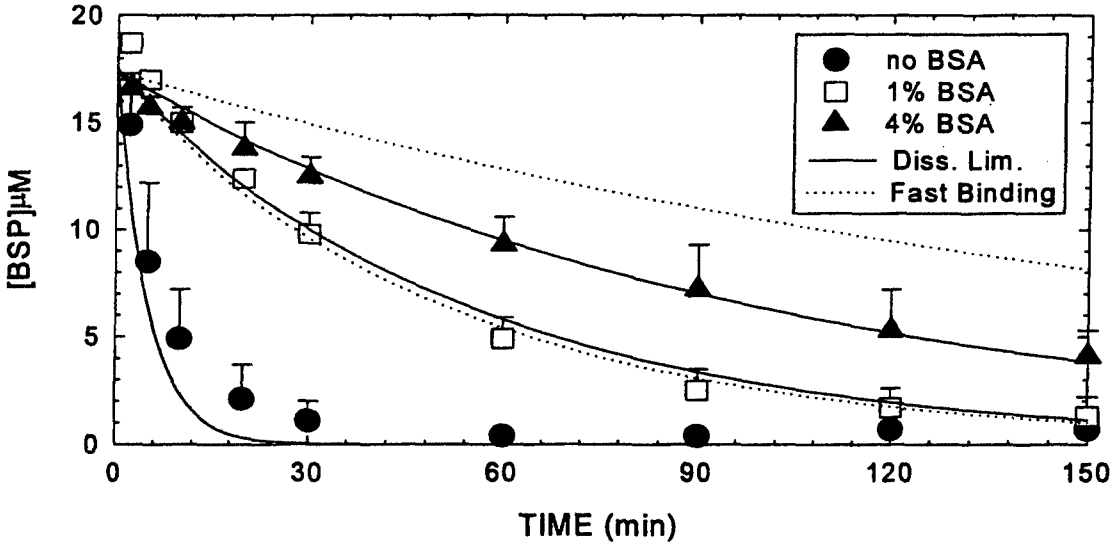

Figure 6.4-1. BSP concentration in perfusion medium.

Rat livers were equilibrated for 30 min in the recirculating IPRL system and then exposed to BSP for 150 min. Initial BSP concentration was 20 µM and the albumin concentration was 0, 1 or 4% (w/v). Results are expressed as the mean ± SD of three separate experiments.

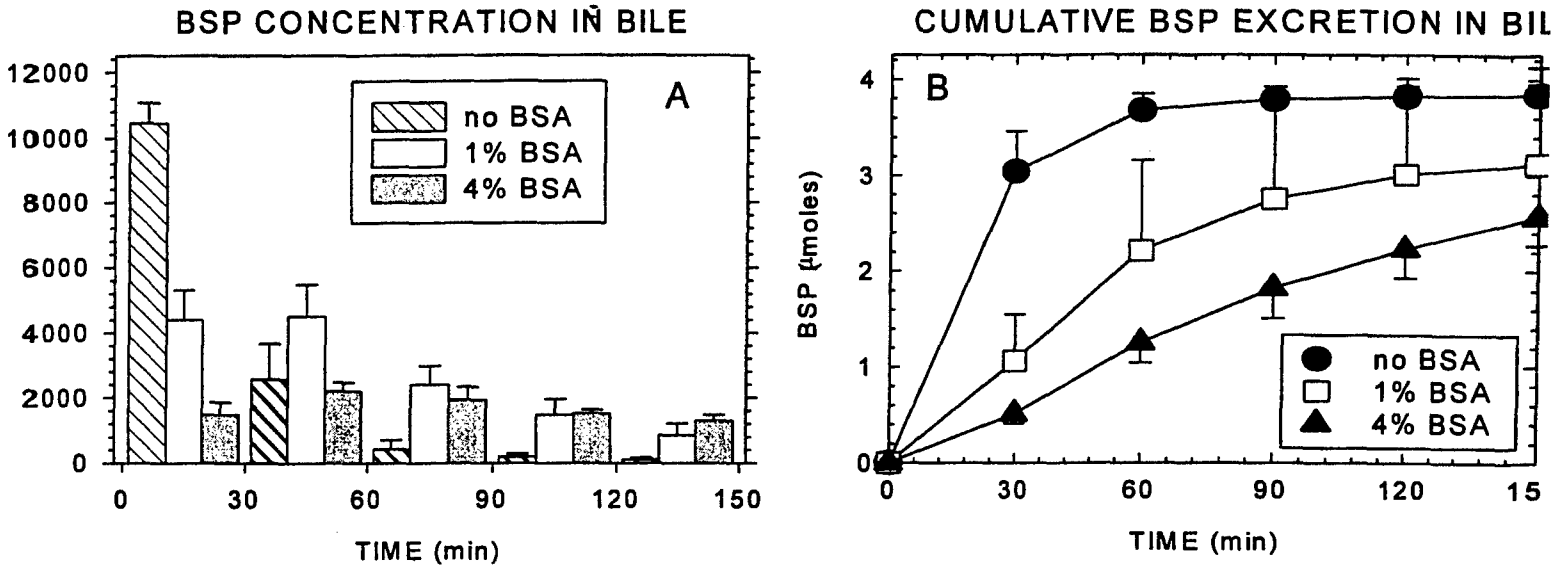

Figure 6.4-2. **(A) BSP concentration in bile.**
(B) Cumulative excretion of BSP in bile.

Rat livers were treated as in Figure 6.4-1.

The BSP concentration in bile during the first 30 min of exposure was high, > 10 mM, in the absence of albumin (Figure 6.4-2A). After the first 30 min, the BSP concentration in bile decreased and was almost zero at the end of the experiment. In the presence of 1% albumin, the bile concentration was 4 mM during the first two 30-min intervals and then declined. In the presence of 4% albumin there were slight changes in BSP concentration in bile. In fact, during the first 30 min of exposure, the BSP concentration in bile was lower than during the following 30-min intervals due to the time delay in collection of excreted bile.

The cumulative excretion of BSP was calculated (Figure 6.4-2B). In the absence of albumin, the cumulative excretion was 3 µmoles, 75% of the initial dose (4 µmoles) in the first 30 min of exposure. Excretion of BSP in the presence of albumin was slower.

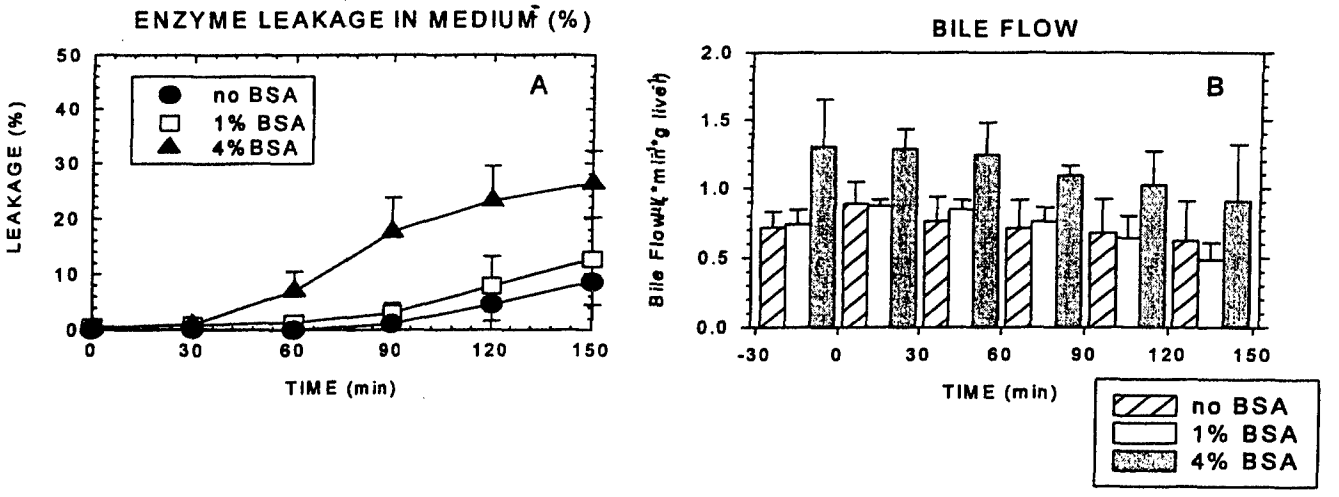

Figure 6.4-3. (A) Percentage LDH activity in perfusion medium
(B) Bile flow in the IPRL ($\mu L * min^{-1} * g\ liver^{-1}$)

Rat livers were treated as in Figure 6.4-1.

The leakage of LDH was higher in the presence of 4% albumin as compared to 1% albumin or in the absence of albumin (Figure 6.4-3A). Enzyme activity in perfusion medium started to increase at t= +30 min in the presence of 4% albumin, whereas it started later in the other treatment groups. The time course of enzyme leakage was similar in the presence of 1% albumin and in the absence of albumin. Bile flow was consistently higher in the presence of 4% albumin (Figure 6.4-2B). There was no difference in bile flow in the absence or presence of 1% albumin.

DISCUSSION

The data presented here clearly illustrate the dramatic effects a change in protein concentration can have on the kinetic behavior of chemicals. The difference in kinetic behavior of 20 µM BSP in the presence of 1% or 4% albumin was profound despite the fact that albumin was in access in both circumstances: 143 µM and 570 µM albumin. Therefore, it is important to consider albumin/protein concentrations when comparing kinetic data reported in literature. A discrepancy in data might be due to different protein concentrations.

When considering the relative importance of 1) membrane transport, 2) metabolism, and 3) protein binding in BSP kinetics, it appears from these experiments, that:

1) The capacity and rate of membrane transport are not rate-limiting factors for BSP uptake and excretion in the presence of albumin. This is indicated by the much higher rate of BSP disappearance in the absence of albumin as compared to its disappearance rate in the presence of albumin (Figure 6.4-1).

2) Although metabolism was not studied in our studies, the very fast uptake and excretion of BSP in the absence of albumin questions conjugation with glutathione as the rate-limiting step for BSP uptake and subsequent excretion. Analysis of bile for BSP and its glutathione conjugate may provide information on whether the ratio of BSP to its conjugate changes in the presence of different albumin concentrations.

3) Protein binding, more specific, the dissociation of BSP from albumin, may be the rate-limiting factor in BSP uptake and excretion. This was suggested by the observation that the rate of BSP uptake was not as elevated at lower albumin concentration as expected based on the assumption of protein binding equilibrium in the sinusoids when curves were fitted for 4% albumin (not shown). This is reflected by the faster disappearance of BSP in the presence of 4% albumin when curves were fit to the disappearance rate in the presence of 1% albumin, assuming binding equilibrium in the sinusoids. The explanation for this phenomenon is that higher albumin concentrations increase the probability of a chemical binding (and dissociating) to albumin (Weisiger et al., 1984). Therefore, at higher protein concentration free chemical may achive equilibrium in its binding with albumin. Recently dissociation limited kinetics in the liver have been reported for indocyanine green (Ott and Weisiger, 1997). The implications of dissociation limited kinetics are especially relevant when extrapolating results obtained with different protein concentrations and assuming protein binding equilibrium in the sinusoids. Uptake is dependent on free ligand but this concentration is limited by protein dissociation.

CONCLUSION

These results illustrate the importance of considering the role of protein binding when studying kinetic behavior of chemicals. Especially when extrapolating *in vitro* data to the *in vivo* situation, the outcome may be affected when protein binding is not correctly taken into consideration, resulting in incorrect chemical concentrations and thus incorrect predictions of toxicological effects.

Acknowledgments

Acknowledgments to Dr. Brent Foy and Frank Dessauer.

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6.5 PREPARATION OF RAT LIVER PLASMA MEMBRANE VESICLES

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ABSTRACT

Plasma membrane vesicles are *in vitro* models used to study transport mechanisms of chemicals. The rat liver plasma membrane vesicle system is being developed in our laboratory as a tool to study liver transport processes. The ultimate goal is to incorporate transport parameters for water soluble compounds in biologically based kinetic models. The relative enrichment in marker enzymes for sinusoidal membrane domain and the canalicular membrane domain were determined. In addition, a preliminary uptake experiment using leucine was performed. The results show that the membrane fraction has a 25-fold increase in sinusoidal marker enzyme activity and a 9-fold increase in the canalicular marker enzyme activity. The experiment with leucine showed that uptake of leucine did take place. Additional experiments are in progress to further characterize the purity of the isolated membrane fraction and to derive transport parameters for leucine in our vesicle system.

INTRODUCTION

Predicting the kinetics of xenobiotics in biological systems is important in the process of risk assessment. Biologically based kinetic (BBK) models have been developed for this purpose. So far most BBK models focused on lipid soluble compounds using partitioning coefficients to describe tissue uptake under perfusion limited conditions. These models do not apply for water soluble compounds such as metals and organic anions, for which the cell membrane forms a barrier for uptake and their distribution is limited by diffusion rather than perfusion. The liver is a major controlling factor in determining systemic kinetics of many chemicals. The specific focus of this project is to investigate the hypothesis that hepatic sinusoidal membrane vesicles can serve as a model system to evaluate transport mechanisms of water soluble compounds (both qualitatively and quantitatively). Data derived from this system can then be utilized in diffusion limited BBK models to predict hepatic kinetics of hydrophilic compounds.

The *in vitro* plasma membrane vesicle model has been used to investigate transport properties of many substrates, e.g., amino acids (Evers et al., 1976), taurocholate (Meier et al., 1984), glucose (Hopfer, 1978; Lever, 1979), and fatty acids (Stremmel et al., 1986). Advantages of using the vesicle system are easy manipulation of the environment (extra- and intravesicular), the lack of metabolizing enzymes, and the lack of binding to intracellular proteins. Furthermore, vesicles can be prepared that are either derived from the sinusoidal region of the plasma membrane or from the canalicular region. This enables description of both uptake and excretion characteristics of ionic molecules or organic acids in BBK models.

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The method chosen by our laboratory to isolate plasma membranes was based on the method described by Prpic et al. (1984). This isolation method utilizes a Percoll gradient, takes less than five hours and has a good yield of liver plasma membranes. First, the enrichment in marker enzymes for sinusoidal plasma membrane and canalicular plasma membrane were determined in the plasma membrane fraction isolated. The marker enzymes were NaK-ATPase for the sinusoidal membrane domain and 5'-nucleotidase for the canalicular membrane domain, routinely used to characterize plasma membrane isolations. To validate the vesicle preparation prepared in our laboratory, it was decided to study the uptake parameters of leucine. There are literature values available for leucine transport parameters using liver plasma membrane vesicles so a comparison can be made between our preparation and those of other groups. The initial uptake study with leucine was performed to gain familiarity with the procedures for performing transport studies.

MATERIALS AND METHODS

Materials

Leucine and EGTA (free acid) were purchased from Sigma Chemical Co. (St. Louis, MO). Sterile percoll was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were of reagent grade. Filters used were type HAWP pore size 0.45 μm (Millipore, Bedford, MA). Filtercount was obtained from Packard Instrument Co. (Downers Grove, IL).

Animals

Male Fischer rats weighing between 230-300 g were used for isolation of liver plasma membranes. Rats had free access to food and water purified by reversed osmosis.

Vesicle preparation

Vesicles were prepared based on the method described by Prpic et al. (1984). After cannulating the portal vein, rat liver was deblooded with ice cold saline (0.9% w/v). The liver was excised and minced in SHE buffer (250 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH 7.4). The liver was homogenized using 10 strokes in a Dounce homogenizer with loose fit and three strokes with a tight fit. After diluting the homogenate with SHE buffer to 6% (w/v), the homogenate was centrifuged for 10 min at 1,464 g. The pellet was weighed and resuspended in SHE buffer to 6% (w/v). Percoll was added to the resuspended pellet (1.4 mL Percoll per 10.4 mL resuspended pellet). After the mixture was centrifuged for 30 min at 34,540 g (Optima™ Ultracentrifuge, Beckman Instruments Inc., Allington Heights, IL), the layer floating near the top was isolated, weighed, and five volumes of ST buffer (250 mM sucrose, 50 mM Tris-HCl, pH 8.0) were added. After resuspending the membrane fraction, the membranes were centrifuged for 10 min at 25,000 g. This wash step removes Percoll which interferes with the

protein assay. The pellet is weighed and four volumes of ST buffer were added. After homogenizing, the suspension was stored at −80 °C.

Biochemical assays

Protein was determined using the BCA protein assay from Pierce (Rockford, IL).

Sinusoidal marker enzyme: Na^+K^+-ATPase

Activity of this enzyme is measured as the fraction P-nitrophenylphosphatase activity that is lost in the absence of K^+ (Scharschmidt et al., 1979).

Canalicular marker enzyme: 5'-nucleotidase

Activity of this enzyme is determined by measuring the release of Pi from 5'-AMP (Widnell and Unkeless, 1967).

Leucine uptake studies (Ruiz-Montasell et al., 1994; Novak et al., 1994; Mosely and Murphy, 1989).
Vesicles were prepared by flushing the membrane suspension through a 25-gauge needle (15-20 times). The experiment was started by adding 20 µL of vesicles (50-75 µg protein) to 80 µL incubation buffer (10mM HEPES/KOH, 125 mM KCl, 0.25 mM $CaCl_2$, 12.5 mM $MgCl_2$, pH 7.5) containing leucine (20 µCi) in test tubes. After the desired incubation period, 3 mL ice cold stopping buffer (100 mM KCl, 100 mM sucrose, 10 mM HEPES/KOH, pH 7.5) was added. The content of the tube was filtered over filters pre-wetted with 3 mL of 1 mM leucine (to minimize non-specific binding of leucine). The incubation tubes were rinsed with 3 mL stopping buffer and the wash was filtered. Filters were rinsed twice with 3 mL stopping buffer and transferred to 7 mL scintillation vials. After adding 6 mL Filtercount, radioactivity was counted for 5 min in a liquid scintillation counter type 2200 CA Tri-Carb (Packard Instrument Co., Downers Grove, IL).

RESULTS

Table 6.5-1 summarizes the recovery of protein, NaK-ATPase activity, and 5'-nucleotidase in the plasma membrane fraction from the initial liver homogenate. Per liver, the protein recovery was 2% (22 mg*liver^{-1}), 43% of the NaK-ATPase, and 15% of the 5'-nucleotidase activity.

TABLE 6.5-1. Protein recovery and specific marker enzyme activities and recovery in liver homogenate and isolated plasma membrane fraction.

Markers	Liver Homogenate	Plasma Membrane fraction	Percent recovery
protein (mg/mL)	8.3	4.3	2
NaK-ATPase (nmol*mg protein^{-1}*min^{-1})	4.1	53.9	43
5'-nucleotidase (mmol*mg protein^{-1}*min^{-1})	3.0	14.2	15

Figure 6.5-1 shows the increase in specific activity of the marker enzymes relative to the specific activity in liver homogenate. There was a 25-fold increase in NaK-ATPase specific activity, the marker enzyme for the sinusoidal membrane domain, and a 9-fold increase in 5'-nucleotidase specific activity, the marker for the canalicular domain of the plasma membrane.

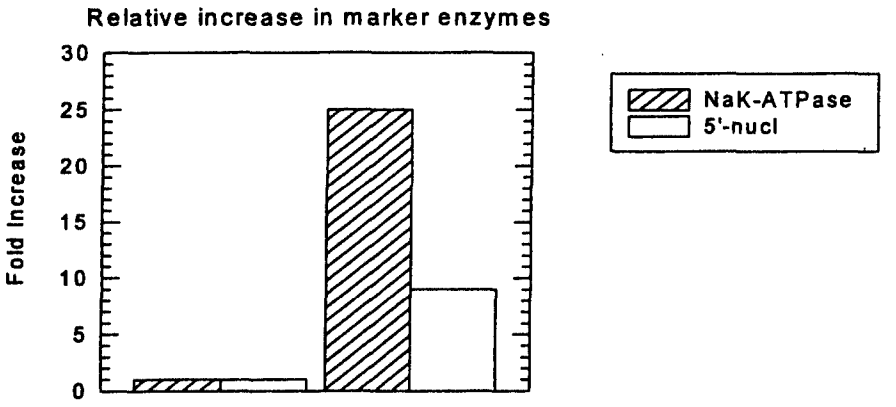

Figure 6.5-1. Relative increase in the specific activity of NaK-ATPase and 5'nucleotidase in plasma membrane vesicles isolated from rat liver. Results are expressed relative to specific activity of these enzymes in liver homogenate.

The preliminary study measuring the uptake of 50 mM leucine shows fast uptake during the first 30 sec of exposure (Figure 6.5-2.). This uptake was slightly higher one hour after the incubation started. The background binding of leucine to the rinsed filters in the absence of vesicles was very low, 0.1% of the total counts.

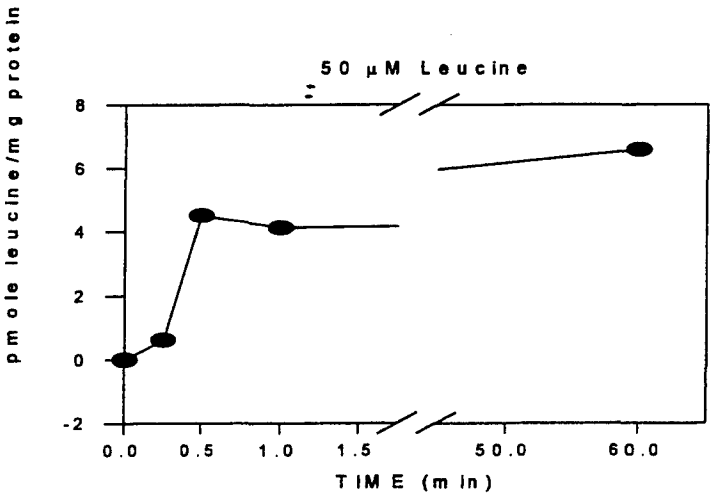

Figure 6.5-2. Uptake of leucine by plasma membrane vesicles isolated from rat liver. Results are expressed as the mean of two incubations.

DISCUSSION

The results presented here describe and illustrate preliminary experiments in the development of a procedure for preparing rat liver plasma membrane vesicles.

The increase in specific activity of the marker enzymes as compared to the original liver homogenate was 25-fold for NaK-ATPase (sinusoidal domain) and 9-fold for 5'-nucleotidase (canalicular domain). These increases are comparable with the increases reported by Meier et al. (1990). They reported an increase of 34 fold for NaK-ATPase and an 11-fold increase for 5'-nucleotidase for their sinusoidal vesicle preparation. Their protein recovery was 0.19 mg/g liver whereas our recovery was 2 mg/g liver. In their canalicular fraction they found an increase of 64 fold for their canalicular preparation and NaK-ATPase activity was absent. The procedure used here yields more membranes per animal, helping in reducing the use of rats, whereas our increases in specific activities are comparable with what Meier et al. (1990) found in their basolateral membrane fraction.

At this moment we are interested in the uptake of chemicals by the liver (transport over the sinusoidal membrane domain) and these results indicate that our preparation is suitable since the sinusoidal enzyme marker is more enriched than the canalicular enzyme marker. It is important to be aware of the presence of canalicular membrane fraction in the preparation when performing uptake studies. However, in most cases it will not pose a major problem since the uptake of chemicals over the sinusoidal membrane in general uses a Na^+ gradient, whereas

canalicular transport is ATP-dependent, to generate the high concentration gradients between bile and sinusoidal medium. Since there is no ATP present in our incubations, it is unlikely that canalicular transporters have a big effect on the outcome of our experiments.

The uptake study indicates that the vesicles are taking up leucine. A fast initial uptake leveling off after 30 sec of leucine in concentrations below 1 mM by liver membrane vesicles was also reported in the literature (Ruiz-Montasell et al., 1994; Novak et al., 1994). More experiments using leucine need to be perfomed before a relevant comparison can be made with values reported in the literature.

In the near future, the functionality of our vesicle preparation will be further investigated using leucine. Furthermore, vesicles will be visualized using electron microscopy and vesicle volume will be determined using radiolabeled glucose. The purity of the plasma membrane fraction will be evaluated by measuring marker enzymes for other cellular organelles, i.e., mitochondria, Golgi, and ER.

CONCLUSION

The isolation procedure is easy, fast, and saves time while developing assays and skills for studies using plasma membrane vesicles. Also, the isolation has a good yield helping to reduce the use of laboratory animals.

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SECTION 7

ADVANCED COMPOSITE MATERIALS PROJECT

7.1 COMBUSTION PRODUCTS FROM ADVANCED COMPOSITE MATERIALS

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ABSTRACT

Recent advances in armament and materials applications are beginning to outpace the development of adequate safety characterizations. To avoid unnecessary and restrictive regulations implemented to protect individuals from potential toxic consequences resulting from exposure to combustion products of advanced composite materials (ACM), this laboratory has begun an investigation of combustion characteristics. In this preliminary investigation we have assessed the production of particulate matter and the production of organic compounds contained in both the combustion vapor phase or associated with the particulate matter. The results of these investigations have revealed that a substantial fraction of the particulates appear to be in the respirable range and that a high number of organic compounds and potential toxicants are associated with particulate matter. These findings are the first to describe the production of potentially toxic atmospheres from the combustion of advanced composite materials and indicate the usefulness of further investigations to quantify the risk of exposure to humans. These and forthcoming data will be useful in determining proper protective equipment and precautions required to protect human health during exposures to products from the combustion of advanced composite materials.

INTRODUCTION

As the U.S. military develops new weapons systems, emerging materials technology is becoming incorporated at earlier stages of development. Just as fiberglass replaced some plastic and metal components in commercial products, composite materials are being developed to replace and augment metal structural components. While composite materials have been available for some time, their composition is being continually refined. The availability of composite materials allows the forming of complex structural components without the risk of fatigue or stress associated with forming such parts from metal. Further, composites offer the advantages of high strength, reduced weight, improved thermoplastic qualities, more controlled thermal expansion, dimensional stability, corrosion resistance, and improved low-observable (stealth) qualities.

Composite materials previously available generally included a reinforcing matrix of continuous fibers stabilized with thermosetting resins, such as epoxy and bismaleimides. Recently, advanced filamentary composite materials have been developed, which substituted carbon filaments for the continuous fibers previously used. Continued

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advances in military weapons systems and their requirements in combination with the advantages of advanced composite materials (ACM) have resulted in the increasing inclusion of ACM in weapons systems.

ACM accounts for 20-25% of the total mass of some presently fielded aircraft, and that figure is expected to rise to near 40% with the further development of additional weapons systems. As components made from these materials become more widely used, the potential for personnel to be exposed to their combustion products increases. The increasing use of ACM in aircraft in general, the recent mishaps with the prototype YF-22 advanced tactical fighter and F-117 "stealth" fighter aircraft, and previously documented illness in firefighters exposed to burning composite materials point out the lack of knowledge about ACM combustion products and the potential consequences for personnel exposed to ACM combustion products. Through the investigation of products released from the combustion of ACM, preliminary data relevant to the protection of human health during an exposure to such products may be generated.

METHODS

Materials

A coarse basket-weave carbon fiber/modified bismaleimide resin (approximate 2:1 ratio by weight) advanced composite material was obtained from an intended aircraft application. ACM was received as "coupons", 108 mm X 108 mm X 2.5 mm and weighing approximately 54 grams. Structure of the generic bismaleimide is presented in Figure 7.1-1.

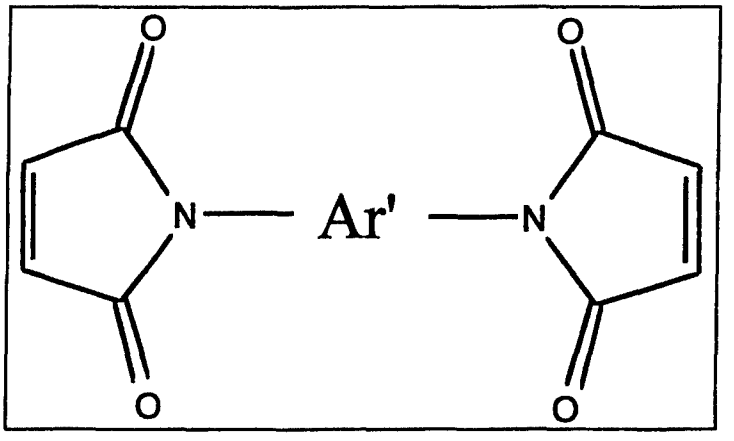

Figure 7.1-1. Generic structure for bismaleimide.

System

A modified UPITT II mini-cone calorimeter (Fire Testing Technology, Inc.) was used to combust ACM (650-880 °C). It included a sample train equipped with negative pressure flow control and embedded sampling devices

(Figure 7.1-2). Mass lost during combustion was measured via a load cell connected to the UPITT II sample platform. A secondary verification was performed before and after the burn via an external analytical balance.

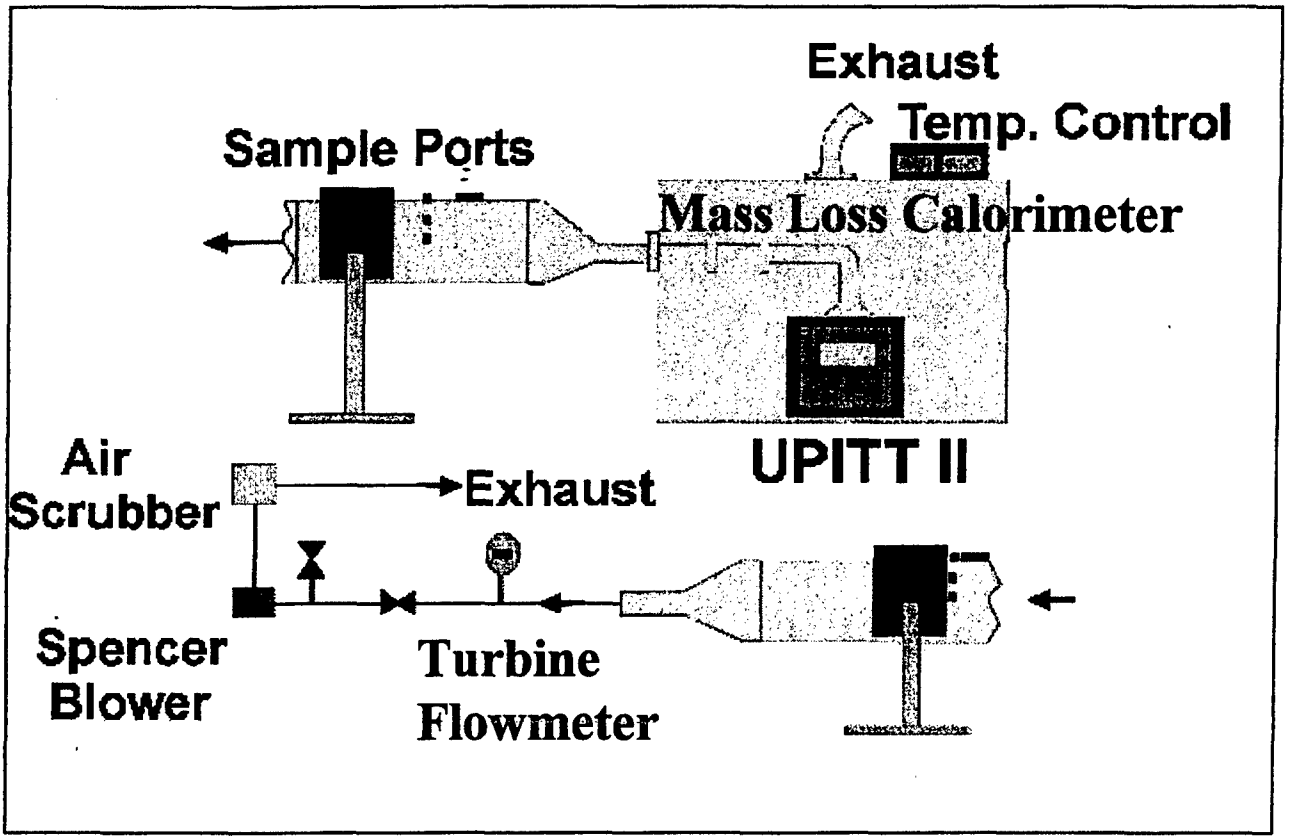

Figure 7.1-2. Combustion system. Sample was heated in the calorimeter and air was impinged through a negative pressure Spencer blower located at the terminal of the plume tube. Sampling devices embedded along the tube allowed the collection and analysis of samples at various intervals along the sample stream.

Sample Collection

Two separate samples of combustion products were collected and analyzed for component identification. The first sample was vapor of the smoke plume which was condensed in a glass impinger immersed in ice water. Air flow through the impinger was 10 L/min for 15 min, a time frame which extended beyond that for sample mass loss. Condensate was extracted with methylene chloride. The impinger was rinsed three times with 5mL of pesticide-grade methylene chloride, and the rinses combined with the extract and concentrated under nitrogen stream prior to analysis. The second sample was particulate matter trapped from smoke on glass wool filters which were in-line with the master pump. Negative pressure air flow resulted in appreciable deposition of particulates, which were then initially extracted with 20 ml methylene chloride:acetone (1:1), then with 100 ml methylene chloride. Passage of particles through thimble pores preempted the extraction of particulate matter via with standard Soxhlet apparatus. The extract was then passed through residue grade sodium sulfate and concentrated under nitrogen stream prior to analysis by gas chromatography/mass spectrometry (GC/MS).

Chemical Analysis

Compounds present in the organic extract of the samples were analyzed by GC/MS. Samples were separated and identified by using a Perkin-Elmer Q-Mass 910 GC/MS equipped with a DB-5 (30m X 0.25 mm, 0.25 μm film thickness) capillary column (J and W Scientific, Folsom, CA). Injector temperature was 270 °C, and an oven temperature program was employed. Starting temperature (35 °C) maintained for 5 min, then increased by 3 °C/min to 120 °C, maintained for 20 min, then increased by 30 °C/min to 300 °C and maintained for the duration. GC effluent was transferred to the mass spectrometer via a heated (220 °C) transfer line. Run time was 170 minutes, and a solvent delay of 4.5 minutes was employed. Separated compounds were identified by analyzing GC effluent over a range from 35 to 650 atomic mass units (AMU) and comparison to the NIST reference library. While the NIST algorithm for location was employed, each match was manually examined for best fit. Preliminary quantification was accomplished by EPA Method 8270 SW846 protocols. Identified components were quantified against nearest internal standards using EPA approximation with a uniform response factor of 1.0.

RESULTS

Mass Loss

The combustion of ACM coupons resulted in flame duration of approximately 5.5 min and an overall loss of 29.0 ± 5.4% mass. Subjective evaluation of the product remaining revealed woven carbon fiber mat, consistent with material safety data sheet composition description.

Particulate analysis

Subjective analysis of trapped particles revealed a fine, black amorphous powder which packed loosely and whose physical characteristics were consistent with elemental carbon. Although our extraction procedure reduced particulate mass by 36.6%, no effort was made to produce a quantitative extraction of compounds. Those compounds extracted by this procedure were subjected to GC/MS evaluation, which identified 90 compounds (Tables 7.1-1 and 7.1-2). The 14 major (of 90 total) extracted components accounted for 60% of the mass of extracted compounds. Data presented in Figures 7.1-3 and 7.1-4 demonstrate the complexity of the mixture and the identification of components. Using data from mass spectra for individual potential combustion products, the total ion chromatogram was searched post hoc and a number of previously undiscriminated peaks were identified, however, their abundance precluded quantitation. They are all low-level EPA target compounds: Indene (peak 6), 2,4-dimethylphenol (peak 8), benzo[a]anthracene (peak 21), benzo[b]fluoranthene (peak 23), benzo[k]fluoranthene (peak 24), 7,12-dimethylbenz(a,h)anthracene (peak 25), and dibenz(a,h)anthracene (peak 26).

TABLE 7.1-1. IDENTIFICATION AND APPROXIMATE QUANTITATION OF MAJOR COMPOUNDS EXTRACTED FROM PARTICULATES

Component[a]		µg/g Particulate	% Weight
Isocyanonapthalene[b]	(14)[c]	3870	0.39%
Quinoline	(10*)	3480	0.35%
Aniline	(2*)	2990	0.30%
1,2-Dihydro-2,2,4-trimethylquinoline (13)		2210	0.22%
Phenol	(3*)	2170	0.22%
Fluoranthene	(19*)	2130	0.21%
Methyquinolines[d]	(12)	1813	0.18%
Anthracene	(17*)	1700	0.17%
2-Methoxyethoxybenzene		1660	0.17%
Dibenzofuran	(15)	1360	0.14%
N-Hydroxymethylcarbazole	(18)	1290	0.13%
Methylanilines[e]	(7)	1216	0.12%
Diphenylether		1050	0.11%

[a]**Only compounds whose combined mass accounted for greater than 1,000 µg/gram particulate are included. These compounds accounted for 60% of the identified extracted mass.**
[b]**Combined 1- (43%) and 2- (57%) isomers.**
[c]**Numbers in parentheses refer to peaks identified in Figure 3, panel A. Asterisk denotes confirmation by internal standard.**
[d]**Combined 3- (20%), 5- (66%) and 8- (14%) isomers.**
[e]**Combined 2- (48%), 3- (51%) and 4- (1%) isomers.**

TABLE 7.1-2. IDENTIFICATION AND APPROXIMATE QUANTITATION OF MINOR COMPOUNDS EXTRACTED FROM PARTICULATES

Component[a]		µg/g particulate	% weight
Tetramethyl-1,4-dihydropyridine[b]		841	0.08%
Pyrene	(20*)[c]	701	0.07%
Fluorene		584	0.06%
Methylisoquinoline[d]		578	0.06%
Indeno(1,2,3-ij)isoquinoline		564	0.06%
Benzo[e]pyrene		550	0.06%
Indole		537	0.05%
2-Ethenylnaphthalene		529	0.05%
Phenanthrene	(16*)	521	0.05%
2-Phenylpyridine		408	0.04%
Methylphenol[e]	(4,5*)	366	0.04%
Chrysene	(22*)	362	0.04%
2-Aminobenzonitrile		354	0.04%
9H-Fluoren-9-imine		352	0.04%

4(1H)-Pteridinone, 2-amino-7-methyl-	286	0.03%
1,1-Diphenylhydrazine	285	0.03%
Dibenzothiophene	280	0.03%
2,4-Imidazolidinedione, 1,3,5-trimethyl-	258	0.03%
Naphthalene (9*)	256	0.03%
2-Phenylnaphthalene	238	0.02%
Benz[e]acenaphthrylene	228	0.02%
Acenaphtho(1,3-B)pyridine	217	0.02%
2-Hydroxybenzonitrile	214	0.02%
2,4,4,6-Tetramethyl-1,4-dihydropyridine	212	0.02%
Benzo[j]fluoranthene	205	0.02%
Benz[a]anthracene, 1,2,3,4,7,13-hexahydro	197	0.02%
Phenylisoquinoline[f]	192	0.02%
Isoquinoline	186	0.02%
9,10,11,12-Tetrahydro-benzo[e]pyrene	183	0.02%
1-Isocyanatonaphthalene	165	0.02%
7H-Benzo[c]fluorene	160	0.02%
N-Phenylacetamide	156	0.02%
9-Methoxyanthracene	153	0.02%
1-Naphthalenemethylphenylacetate	152	0.02%
1-Methyl-4-phenylpyridinium	132	0.01%
1,1'-Biphenyl-2-carbonitrile	130	0.01%
Phenanthrene, 4-ethyl-5-methyl	124	0.01%
Naphthacene	123	0.01%
5-Methylindolizine	121	0.01%
9-Phenanthrenecarboxaldehyde, O-acetyloxy	117	0.01%
1,1-Diphenylhydrazine	110	0.01%
2-(Phenylethynyl)pyridine	103	0.01%
5-Benzoquinoline	98.3	0.01%
5H-Indeno[1,2-b]pyridine	87.6	0.01%
1-(Phenylmethylene)-1H-indene	81.6	0.01%
1H-Phenaline	71.1	0.01%
1-Methylnaphthalene[g] (11*)	66.4	0.01%
4,8-Dimethylisoquinoline	56.5	0.01%
9-Methylacridine	48.9	0.00%
3-Methylphenol	46.1	0.00%
2-Propenenitrile,3-phenyl-, (E)-	40.5	0.00%
2-Aminoisocyanate	39.3	0.00%
1,2-Dihydrocyclobuta[b]quinoline	38.9	0.00%
2-Methyl-N-phenylaniline	30.7	0.00%
N-(1-Phenylethylidene)-methanamine	25.2	0.00%
4-Methyl-1,1'-biphenyl	19.6	0.00%
4-Methyl-1-isocyanobenzene	15.2	0.00%
Dimethylquinoline[h]	14.4	0.00%

4-Aminostyrene	13.4	0.00%
Benzene, (2,4-cyclopentadien-1-ylidene)methyl	3.2	0.00%
5-Methylpyrimido[3,4-a]indole	2.8	0.00%
3-Methyl-1-isocyanobenzene	2.7	0.00%
Diphenylmethane	0.9	0.00%

[a]72 components accounted for 40% of the identified extracted mass.
[b]Combined 1,2,4,6- (75%) and 1,4,4,6- (25%) isomers.
[c]Numbers in parentheses refer to peaks identified in Figure 3, panel A. Asterisk denotes confirmation by internal standard.
[d]Combined 1- (41%), 3- (35%) and 7- (24%) isomers.
[e]Combined 1- (43%) and 2- (57%) isomers.
[f]Combined 4- (33%) and 8- (67%) isomers.
[g]Combined 1- (50%) and 2- isomers.
[h]Combined 4,8- (83%) and 5,8- (17%) isomers.

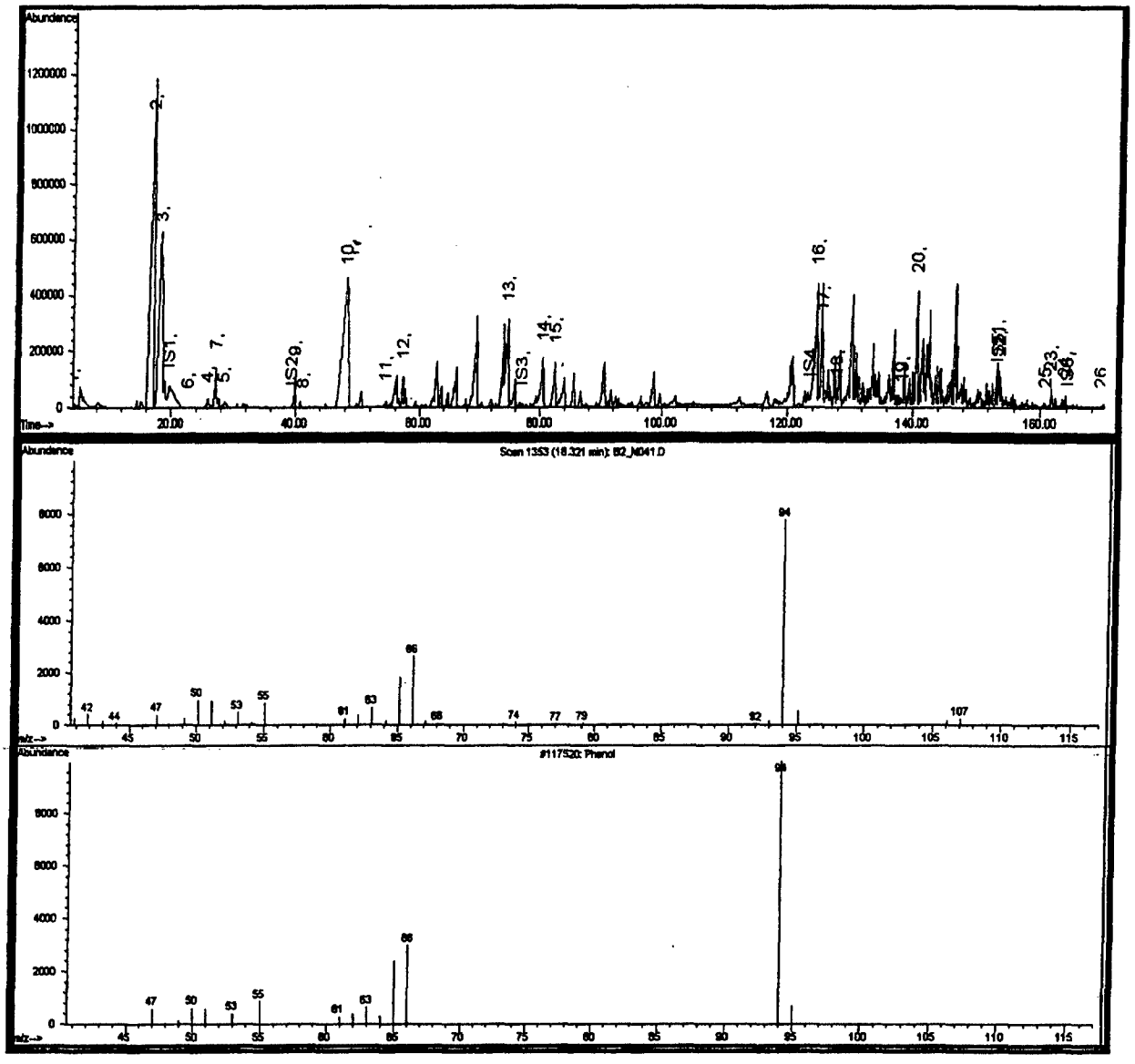

Figure 7.1-3. **Combustion products from advanced composite materials. Panel A shows the total ion chromatogram of products released during the combustion of an ACM sample. Panel B shows the ion chromatogram of a single peak (peak 3, retention time 18.321 minutes), and panel C demonstrates the closest NIST library match for that component, phenol. Numbered peaks can by identified by numbers in parentheses in Tables 7.1-1 and 7.1-2 (to avoid unnecessary distraction, not all peaks were numbered). Internal standards (IS-1 to IS-6), retention times of 20, 40, 75, 125, 155, and 165 minutes are 1,4-dichlorobenzene, d4, naphthalene, d8, acenaphthene, d10, phenanthrene, d10, chrysene, d12 and perylene, d12, respectively.**

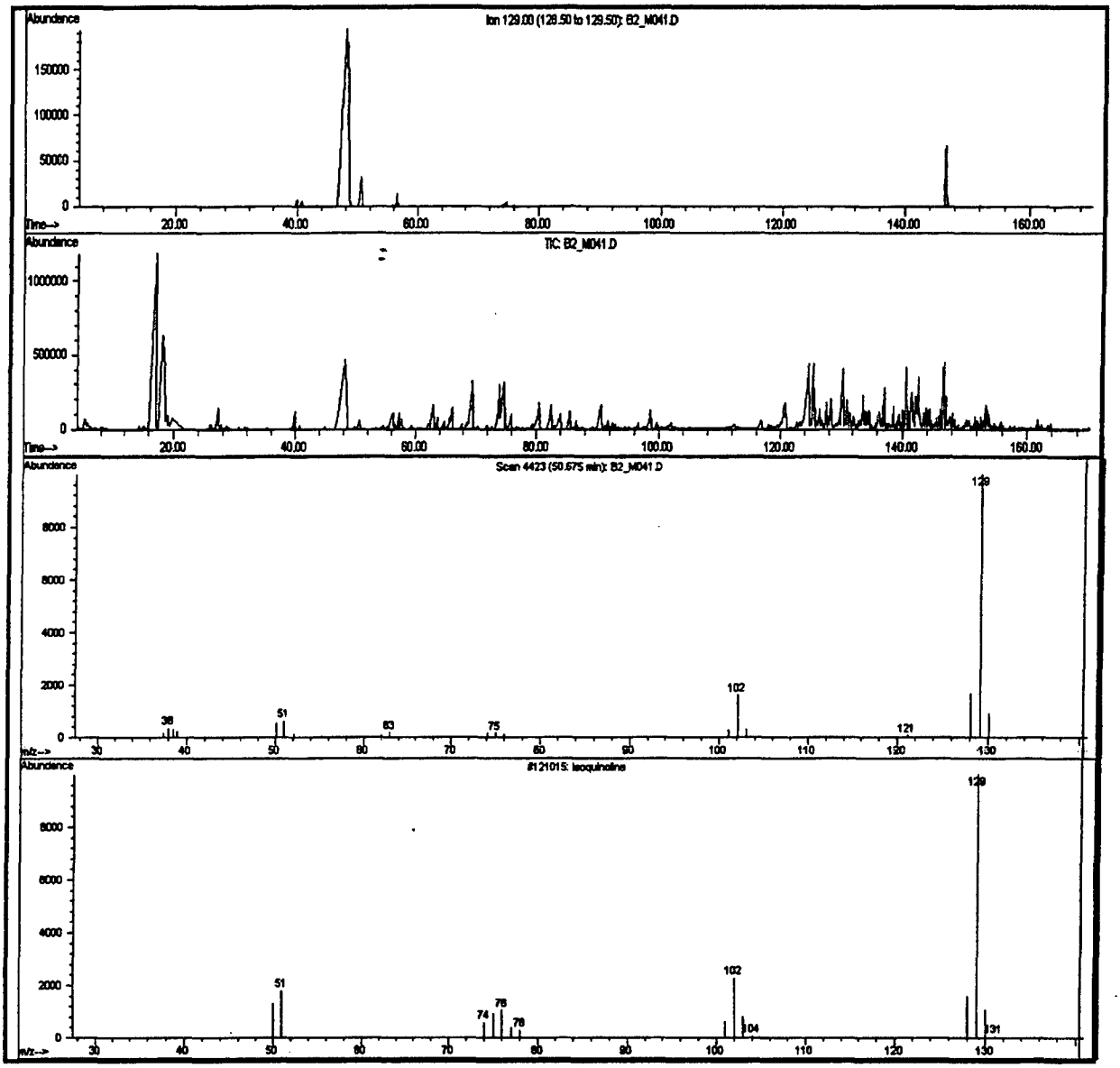

Figure 7.1-4. **Resolution of isomeric forms. Panel A depicts the extracted ion chromatogram for mass 126, the characteristic mass for both quinoline and isoquinoline, above the total ion chromatogram, note peaks at approximately 50 mins retention time. Panel B represents the ion chromatogram for the peak at 50.675 minutes (peak 10, Figure 7.1-3), and panel C represents the library standard for isoquinoline. Because the authentic standard for quinoline (peak 10) indicated an ion chromatogram identical to those for both quinoline and isoquinoline but indicated a retention time of 50 minutes and no following peak, we interpret these findings to indicate that quinoline is the predominant form and isoquinoline a product of lesser magnitude.**

Identification of structurally similar compounds

The resolution of isomeric forms was accomplished by either of two methods. Approximately 20 authentic standards were employed to determine component retention times. Those compounds in Tables 7.1-1 and 7.1-2 whose retention times and identities were confirmed by authentic standards are identified by an asterisk. Because

the mass spectra for quinoline and isoquinoline are identical, retention time of authentic compound and the lack of trailing peak of identical mass spectrum was used to identify quinoline as the predominant form (Figure 7.1-4). An alternate method was used to differentiate isocyanonaphthalene from isocyanatonaphthalene. Since isocyanatonaphthalene has an extra oxygen in its structure, the evaluation of mass spectra can be used for differentiation of from isocyanonaphthalene. To confirm isocyanonaphthalene and rule out the toxicologically different isocyanatonaphthalene, the mass spectra were evaluated. Because the base peak in the mass spectrum for Figure 7.1-3's peak 14 matched that for isocyanonaphthalene (153 AMU) and not that for isocyanatonaphthalene's ion spectrum base peak at 169 AMU (Figure 7.1-5) we conclude that the compound is isocyanonaphthalene.

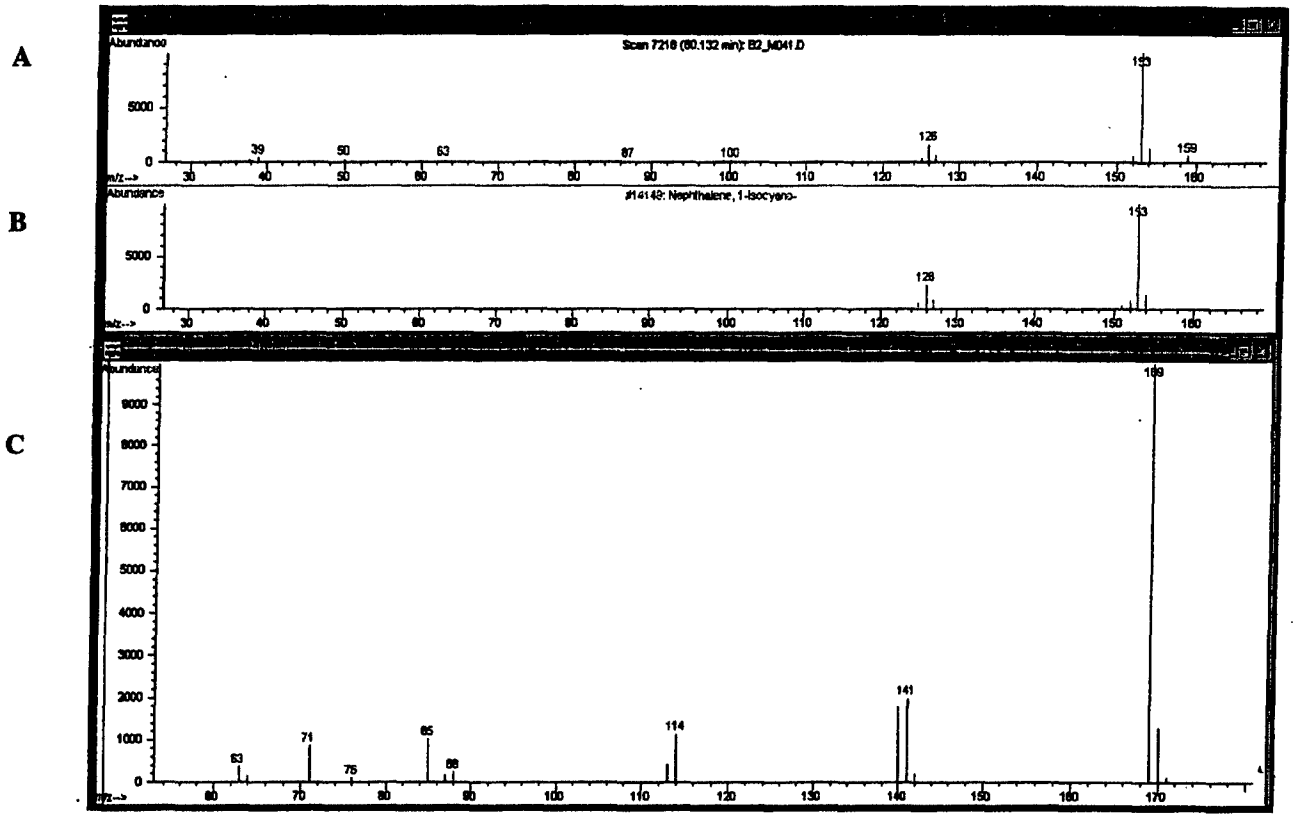

Figure 7.1-5. Identification of isocyanonaphthalene. Panel A represents the ion spectrum for peak 14, base peak at 153 AMU and retention time of 80.132 minutes. Identification as isocyanonaphthalene was accomplished by comparing the spectrum to that for authentic isocyanonaphthalene, base peak at 153 AMU (panel B) and to that for authentic isocyanatonaphthalene, base peak at 169 AMU (panel C).

Vapor analysis

The eight components identified in condensed vapor (Table 7.1-3) accounted for 90+% of the area counts in the total ion chromatogram. The other 10% of total area counts were indistinguishable from background, as no additional major peaks were identified by our analytical technique.

TABLE 7.1-3. APPROXIMATE CONCENTRATIONS OF VAPOR COMPONENTS

Compound[a]	Air Conc. (ug/m^3)
Phenol	1600
Aniline	571
Methylphenols[b]	231
Diphenylether	190
Quinoline[c]	41.5
Biphenyl	11.4
3-Isocyanotoluene[d]	9.8

[a]Results are presented as approximate quantity recovered from the condensation of 150 vapor from combustion of ACM coupons.
[b]Combined 2- (54%) and 4- (46%) isomers. The 3-isomer was not identified in condensate.
[c]By comparison, quinoline was the single major component identified in particle extract.
[d]No isocyanotoluene isomers were identified in particulate extract.

DISCUSSION

Recent advances in weapons system development have outpaced the development of relevant safety guidelines. The inclusion of composite materials is one example where combustible components are included in systems already fielded or under development. Because the materials replaced by composites were generally benign (metals), system developers are not yet accustomed to determining toxicity evaluations on structural components. This report is the first to identify the toxic potential of composite combustion exposures and was conducted as a preliminary investigation to determine which broad classes of compounds might be expected from the combustion of ACM. As an initial screen, no attempts at quantification were undertaken. Because mishaps involve entire aircraft, toxic potentials for compounds released from the combustion of other components (rubber, plastic, fuel, lubricants, and hydraulic fluids, insulation, etc.) should be included in a comprehensive analysis of risk for exposure to products from entire airframes.

In the present assessment we have evaluated the combustion-induced disposition of a form of ACM which is intended for use in advanced aircraft. The goal of this study was to obtain a preliminary (qualitative) identification of materials produced from combustion of ACM. As such, we focused on the volatile compounds which were released following combustion and minimal particulate analysis. Our data revealed that 70% of the ACM coupon's mass remained as a carbon-fiber based material following combustion. Subjective analysis indicates that this mass likely represents the fibers which are embedded in the epoxy binder of the composite, consistent with characteristics identified in the MSDS. The other 30% was combusted to carbon monoxide, carbon dioxide, water, and other material-specific products of combustion or was lost as particulate matter. Because we did not employ a quantitative assessment of organic-extractable components, our data represent a preliminary examination of some of the products identified from the combustion of this ACM sample.

Without the molecular structure and cross-linking information for this bismaleimide composite, we can only speculate on the mechanisms of formation for the complex mixture of compounds produced from the combustion of this ACM sample. The bulk of these compounds are represented by polycyclic aromatic hydrocarbons, anilines, phenols, and quinolines. However, the analysis of products demonstrates some trends. The lack of demonstration of any maleimide moieties is evident. Even though the data were searched post hoc using the mass spectrum from the library standard for maleimide, no matches were found among the peaks detected. This may indicate the destruction of that moiety and the molecular rearrangement of its components, which might contribute to the finding of homologous series of compounds. These compounds may undergo a series of free radical reactions, as might occur during combustion. In a free radical environment, smaller molecular fragments collide to form higher members of the series. This is best demonstrated by the finding of phenol, cresols (methylphenols), and dimethyl phenol compounds. The aromatic nature of these compounds confers resonance stabilization and may contribute to presence as relatively stable combustion products. Further, the large quantity of soot formed is consistent with incomplete combustion of aromatic compounds, most of which produce significant amorphous carbon, such as lampblack. Condensation of organic compounds, as evidenced herein, may obscure the electron microscopic resolution of the underlying particulate structure.

Analysis of organic-extractable components associated with particles revealed 90 chemicals produced from the combustion of ACM. The distribution of combustion products to respirable particles will alter the pulmonary deposition of products. These particle-associated products may be respired and delivered to end-respiratory tissues (alveoli). Quinoline is one combustion product identified in extract of both condensed vapor and particles, but it accounts for much more of the extractable mass of particles (8.7% of extractable mass) than in the vapor phase (1.6% of extractable mass). This may indicate that particles are the major transport mechanism for quinoline. The condensation of quinoline (BP = 238°) onto available particles is likely responsible for the high degree to which this and other high-BP compounds become associated with particulates. The adsorbance of toxicants onto particulate matter has been demonstrated to alter pulmonary deposition, retention time, and toxicity. This phenomenon should be included in any exposure assessment for quinoline following ACM combustion.

We have identified several compounds which are mutagenic or carcinogenic in animals. Quinoline is a major ACM combustion product which is also found in tobacco smoke and has been demonstrated to produce DNA damage. Exposure to quinoline may alter the function of the retina and optic nerve, cardiovascular system, and central nervous system. N-hydroxymethylcarbazole, also, has been demonstrated to be a mutagen. While aniline, also, is carcinogenic and can produce methemoglobinemia, past studies on aniline dye workers have established a link between exposure and risk to bladder carcinoma only at high exposures. The elimination of aniline in urine and its recrystalization and sedimentation in the bladder result in irritation of the epithelium and resulting

carcinoma. Biomedical effects of toluidine include CNS depression, cyanosis, methemoglobinemia, vertigo, headache, and confusion. The likely effects of exposure to aniline (BP = 185°) and 2- and 3- methylaniline (toluidine, BP = 195°) under the conditions surrounding a combustion-related exposure may be predicted to include CNS depression and methemoglobinemia. Like the aniline compounds, phenol (carbolic acid) is rapidly absorbed from inhalation exposures and may cause acute symptoms including shock and CNS depression. Although phenol's primary target system is the CNS, other effects include methemoglobinemia, Heinz body hemolytic anemia, and pulmonary edema. Diphenyl ether is released during the manufacture of plastics and from turbine engines, as well as being used in soaps, detergents, and as a dye carrier in the textile industry. It is not a serious hazard as a mutagen, but acute exposures in rodents have produced injury to the spleen, liver, kidney, thyroid, and intestinal tract. Anthracene itself has not been classified as carcinogenic, although many of its derivatives commonly found in coal tar, tobacco smoke, and petroleum products are potent carcinogens. The acute toxicity (bronchitis) associated with exposure to anthracene is presumably due to derivatives. Fluoranthene is released to the environment from the general combustion of organic matter and fossil fuels and is photolyzed with a half life of 4-5 days. Effects of acute exposure include tachycardia and cardiac arrhythmias, nausea, liver injury, pulmonary edema, and respiratory arrest. Although not classified as a human or animal carcinogen, NIOSH has recommended it be regulated as an occupational carcinogen. Finally, dibenzofuran is also a product of the combustion of coal and petroleum products and not considered carcinogenic for animals or humans, but exposure to high concentrations can produce chloracne. Its presence in the gas phase seems transient, with a half-life of 11 h, but association with particulate matter may prolong its presence in the atmosphere.

It is important to compare the implications of toxicity from this limited-scope evaluation of ACM combustion to the scenario involving the complete aircraft. Adequate toxicity information exists for few of the major ACM combustion products. For many more, structure-activity relationships may help predict their toxic potential. This preliminary study identified three main classes of combustion products: nitrogenous aromatic compounds, polynuclear aromatic hydrocarbons (PAHs), and phenols. The complex mixture of compounds and the degree of exposure complicate predictions of latency, duration and extent of resulting toxic consequences following exposure to ACM combustion products. As with any exposure to burning materials, proximity correlates well with toxicity. The use of personal protective equipment (PPE) may abate the dangers associated with products of combustion, but adequate PPE may not be available. Due to the relative distribution of compounds to particulates, it would seem that (in the absence of other PPE) particle filtration masks may afford some measure of protection. To further quantify the toxic potential of material-specific combustion products, efforts should focus both on individual components and the complex mixture.

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7.2 POTENTIAL HEALTH HAZARD ASSOCIATED WITH COMBUSTION OF ADVANCED COMPOSITE MATERIALS

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ABSTRACT

Use of new synthetic materials, such as advanced composite materials (ACM), has raised concern regarding the potential human health risks resulting from exposure to the chemically complex smoke produced by burning material. We developed an analytical protocol for evaluating the smoke produced from controlled combustion of test materials in a modified cone heater combustion module of the UPITT II apparatus. The objective of this study was to sample soot and aerosol for chemical analysis, morphological evaluation, and identification of organic compounds. The results demonstrated that 43% (1463/3430) of the particles produced in the smoke were of respirable aerodiameter. The particle morphology was an amorphic carbon rather than fibrous material. We identified over 90 compounds which is consistent with other polymer combustion studies. Quinoline, aniline, and phenol were found in both the vapor and soot fractions. The risks associated with combustion of ACM include inhalation of particulates and organic vapors.

INTRODUCTION

Advanced composite materials (ACM) offer substantial weight savings in airframes, ships, submarines, and armored vehicles. Numerous military aircraft, including fighters, bombers, and Harriers contain varying amounts of ACM. The ACM matrix is created by assembling two or more physically distinct synthetic components, reinforced (graphite) and bound with resin (epoxy), integrated into various configurations to obtain specific characteristics, and properties. However, the replacement of nonflammable materials (metals such as aluminum) with organic binder resins contributes to fire involvement, which requires consideration of heat fluxes.

As a result of wheels-up mishaps involving ACM aircraft, concerns have been raised over the data gaps on safety and health risks of burned or burning composite materials. The discovery of dead birds around the partially burned aircraft and worker complaints suggest the possibility that toxic off-gassing of the burned composites occurred during and long after the combustion ceased. In response to concerns voiced at the October 1991 Horizons Conference on Health and Safety Concerns of ACM at Wright-Patterson AFB, we initiated an effort to characterize and assess the by-product from burned composites. System Program Offices for aircraft with ACM, ACM Laboratory (McClellan AFB) and US Air Force Surgeon General's Office also expressed concern over the health impacts from burned ACM. Although ACM appears to present limited or no human health hazard in its original state, the

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combustion products, as well as possible graphite fibers, are not well characterized. Over 90 chemicals were identified from initial combustion studies of ACM.

Traditional risk assessment paradigm includes hazard identification, dose-response assessment, exposure assessment and risk characterization. We have attempted to apply some of the same principles to combustion studies while incorporating an interdisciplinary approach of toxicology, bioenvironmental engineering, chemistry, and image analysis. In this effort we focused on particle size and morphology, soot composition and volatile organic compounds to provide a preliminary identification of potential health hazards and recommended controls.

MATERIALS AND METHODS

A modified UPITT II cone calorimeter to permit control of heat flux and airflow was used to burn ACM under various test parameters for flow and temperature. Two samples of combustion products were collected and analyzed in a Perkin-Elmer 910 GC/MS (Gas Chromatograph/Mass Spectrometer) system. The first sample was a vapor from the smoke plume (produced at a temperature of 880 °C and flow of 400 L/min) captured in a cold trap, extracted with methylene chloride, and concentrated with a stream of dry nitrogen. A total of 150 liters (10 L/min for 15 min) of the combustion gas was drawn through the trap. The second sample of deposited soot, combined from several runs, was collected on a glass wool filter taken at a site 8ft from the source. The standard method for extraction (Soxhlet) could not be employed due to the small particle sizes. Instead, a glass pipette plugged with glass wool was used. The extract (50:50 methylene chloride:acetone) was concentrated to approximately 1 mL by a stream of dry nitrogen and then made up to 20 mL with methylene chloride. Compound identification was performed quantitatively using the latest available National Institute of Standards and Technology reference library and approximate quantification was performed under modified EPA Method 8270 protocols.

Two types of particulate samples were collected for examination by scanning electron microscope (SEM) and light microscope. Samples for evaluation by SEM were dried overnight in a vacuum desiccator, then sputter-coated with a 10-15 nm layer of gold. Photomicrographs were taken of the surfaces using an Amray 1000B SEM at 20-30 kV accelerating voltages. The second sample type, using glass microscope slides placed adjacent to the SEM stubs, was collected for surface aerosol particles. Samples collected on glass microscope slides were magnified to 40x, 200x and 400x by a light microscope and the resulting images captured by a microscope-mounted Charged-Coupled Device camera and digitized for image analysis. Algorithms developed for particle analysis were employed using a Leica Quantimet 570c image analysis system. Particles were detected as "features" in each calibrated digitized image by comparing the gray level of the feature with the background gray level. The identified features were then measured using computer-based image analysis methodologies for area, perimeter and equivalent circle diameter .

RESULTS

Both temperature and flow influenced particle size as shown in Table 7.2-1. Particle size significantly differed among the temperatures. At 770 °C and the highest flow rate of 650 L/min the percent (68.9%) of particles which was greater than ten microns was significantly different than those at other temperatures. At a temperature of 880 °C and flow of 650 L/min, the percent (53.5%) of particle size which were less than one micron was significantly different also. All three temperatures showed significant differences in particle size among the flow rates. Regardless of the flow and/or temperature, 42.7% of all particles produced were equal or less than 5 microns. Scanning electron micrographs showed amorphous particles of various sizes, most significant were those of the respirable portion. No fibers were identified in the soot or aerosol particulate samples collected at any of the temperatures or flows. Our data revealed that the majority (50-75%) of the actual ACM remained behind following combustion as a carbon fiber-based material. This presumably represents the fibers which are embedded in the epoxy binder of the composite. The balance of the material was combusted to CO, CO_2, water, and other material-specific products of combustion.

Soot and vapor were analyzed for compound identification by mass spectroscopy. Of the volatiles trapped in solvent and analyzed by gas chromatography, eight major constituents accounted for over 90% of the volatile compounds. Of the soot recovered, approximately 30% of the mass was extractable and identifiable by gas chromatography. The results of this examination identified over 90 individual compounds. Subjective analysis of the soot revealed a fine, black amorphous powder which packed loosely (1.5 grams occupied 5 mL, where 1.5 grams water would occupy 1.5 mL) and was consistent with elemental carbon. The major compounds identified for this assessment are listed in Table 7.2-2 based on percent weight of the soot at the collection site and Table 7.2-3, approximate concentration of volatile compounds. These four constituents, found in both soot and vapor, account for 22% of the mass extracted from the soot and over 50% of the area counts in the total ion chromatogram for the 90+ compounds identified. Of the major compounds identified aniline, phenol and quinoline were considered to be of toxicological importance.

DISCUSSION

In this assessment we evaluated the combustion-induced characterization of particles from the ACM intended for use in advanced aircraft. The goal of this study was to identify potential health hazards produced from combustion of ACM. As such, we focused on two areas: 1) the volatile compounds which were released following combustion, and 2) the nature of any particulate material released through combustion.

These analyses identified nearly 90 chemicals produced from the combustion of ACM and approximately 30% of the starting mass was vented during combustion. The initial analysis of data collected showed that approximately 43% of the particles in the smoke had an aerodynamic diameter equal to or less than 5 microns (5μm) of which

34.5% were equal to or less than 1 micron (1μm). The implication of this finding is that the inhalation hazard may be substantial for the combustion products of ACM, given the complex organic composition of the smoke and the small particle size. Particles less than 5 um have the potential for deposition within the respiratory tract via diffusion and sedimentation. The greatest significance is amorphic carbon particles $\leq$ 1 um diffusing in the alveoli. We clearly identified chemical products of ACM on these particles. The presence of these potentially dangerous chemicals associated with the particulate matter raises the probability of deposition within the alveoli and augments the potential for health hazard(s). This may result in increased potential for toxic consequences. Of equal significance is the lack of fiber particles in the combustion products and would therefore suggest that a fiber inhalation hazard does not exist.

Hazardous chemicals identified of significant toxicological concern were quinoline, aniline and phenol. Quinoline is known to have carcinogenic and mutagenic activity in laboratory animals and exposure may affect cardiovascular and central nervous systems. Aniline causes methemoglobinemia and along with its homologues has been associated with bladder cancer. Acute exposure to phenol may result in shock, hypotension, methemoglobinemia, Heinz body hemolytic anemia, and central nervous system depression. Based on the compound with the lowest LD_{50} (aniline), in an occupational exposure scenario, the Threshold Limit Value-Time Weighted Average for aniline was not exceeded. However, the outcome of exposure to a mixture of chemicals and primary irritation by other combustion products was not predicted or accounted for in this assessment. Combustion studies by Levin demonstrated additive and antagonist effects from inhalation exposure to combusted polymers. Therefore, dose-response effects may not provide the largest margin for safety when applied to combustion of ACM.

The route of primary exposure was determined to be by inhalation and the target receptors to be aircrew, fire fighters and ground personnel conducting disaster response and cleanup operations. After a fire is contained then the dermal route may also be a concern. Phenol and quinoline are both considered to have potential significant cutaneous absorption, as well as via mucous membranes.

Toxicity of ACM combustion products using a bioassay has been developed and will be completed in the following effort. However, preliminary results have indicated cyanide gas is released at significant levels. Specific biological and physiological parameters of the bioassay will be evaluated for acute health outcomes. Until those results are available biomarkers and biological effects monitoring should be considered for any individuals exposed to ACM combustion products. Urine samples can be tested for aniline, total p-aminophenol, and total phenol. Blood tests can be conducted for methemoglobinemia, Heinz bodies and hemolytic anemia. Exposed individuals may exhibit cyanosis, central nervous system and cardiovascular system depression, hypotension, gastrointestinal cramping, pulmonary edema, and vertigo. Prevention of possible exposure is always the preferred method for controlling risk. Personnel protective equipment to address inhalation of both organic and

particulate hazards, as well as proper gloves and clothing to prevent dermal exposure is recommended when exposed to the combustion products of ACMs.

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TABLE 7.2-1. THREE-WAY INTERACTION FOR TEMPERATURE (°C)/FLOW (L/MIN)/INTERVAL(μm)

FLOW	TEMPERATURE	INTERVAL	COUNT	PERCENT
340	625	0≤μm≤1	329	37.8
	625	1<μm ≤5	67	7.8
	625	5<μm ≤10	4	0.4
	625	> 10μm	471	54.1
	770	0≤μm≤1	84	19.4
	770	1<μm ≤5	12	2.8
	770	5<μm ≤10	2	0.2
	770	> 10μm	336	77.4
	880	0≤μm≤1	162	33.8
	880	1<μm ≤5	32	6.7
	880	5<μm ≤10	3	0.6
	880	> 10μm	283	59.0
370	625	0≤μm≤1	94	30.1
	625	1<μm ≤5	27	8.6
	625	5<μm ≤10	5	1.5
	625	> 10μm	186	59.6
	770	0≤μm≤1	93	30.9
	770	1<μm ≤5	31	10.3
	770	5<μm ≤10	1	0.3
	770	> 10μm	176	58.5
	880	0≤μm≤1	166	38.6
	880	1<μm ≤5	35	8.2
	880	5<μm ≤10	7	1.6
	880	> 10μm	222	51.6
400	625	0≤μm≤1	161	50.2
	625	1<μm ≤5	48	14.9
	625	5<μm ≤10	3	0.9
	625	> 10μm	109	34.0
	770	0≤μm≤1	93	33.1
	770	1<μm ≤5	29	10.3
	770	5<μm ≤10	8	2.9
	770	> 10μm	151	53.7
	880	0≤μm≤1	93	31.5
	880	1<μm ≤5	29	9.9
	880	5<μm ≤10	2	0.6
	880	> 10μm	171	58.0
650	625	0≤μm≤1	306	44.2
	625	1<μm ≤5	38	5.5
	625	5<μm ≤10	3	0.4
	625	> 10μm	345	49.9
	770	0≤μm≤1	107	26.4
	770	1<μm ≤5	18	4.4
	770	5<μm ≤10	1	0.2
	770	> 10μm	279	68.9
	880	0≤μm≤1	267	53.5
	880	1<μm ≤5	31	6.2
	880	5<μm ≤10	4	0.8
	880	> 10μm	197	39.5

Three-Way Interaction; X^2 = 152.29; df = 54 p = 0.0000

TABLE 7.2-2. IDENTIFICATION AND APPROXIMATE QUANTITATION OF MAJOR COMPOUNDS EXTRACTED FROM COMBINED SOOT SAMPLES

Identified Compound	Concentration in Soot (μg/g)	Concentration in Soot (% Weight)
Aniline	2990	0.30%
Phenol	2170	0.22%
Quinoline	3480	0.35%
Diphenyl Ether	1050	0.11%

TABLE 7.2-3. APPROXIMATE CONCENTRATIONS OF VAPOR COMPOUNDS AT 880 °C AND FLOW OF 400 L/MIN

Compound	Air Conc. (μg/m3)
Aniline	571
Phenol	1600
Quinoline	41.5
Diphenyl Ether	190

7.3 ADVANCED COMPOSITE MATERIAL COMBUSTION MODEL

C.D. Flemming, K.J. Kuhlmann, J.C. Lipscomb, and D.L. Courson

ABSTRACT

Advanced Composite Materials (ACM) have the desired properties (i.e. improved thermoplastic properties) to be used in the manufacturing of aircraft. Since composite materials are new, there is an interest in the potential short-term toxicity effects (incapacitation and lethality) and long-term effects (injury to lungs and other target organs) to airplane personnel and firefighters who will be exposed by-products of ACM combustion.

A preliminary model was developed in FORTRAN since it involves integrating the estimation of short-term (time to hazardous conditions, toxic potency, incapacitation and lethality) and long-term effects (estimation of gas concentration using principal component regression, a physiological model and dose-response using logistic regression).

The dose-response is used to estimate the risk of certain toxic effects. The FORTRAN compiler was chosen since the model required the use of numerical analysis and an existing FORTRAN subroutine package. FORTRAN has a long history in processing of scientific data, and can easily be modified as the sophistication of the model develops. No statistical package was used except to validate the FORTRAN code.

INTRODUCTION

Advanced composite materials (ACM; Figure 7.3-1) are lightweight, have high strength and improved thermoplastic properties (rigidity, conductivity, and thermal expansion).

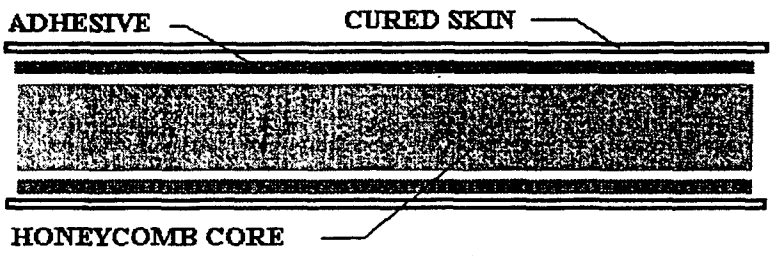

Figure 1. Typical Cross-Section of Advanced Composite Material

FIGURE 7.3-1. TYPICAL CROSS SECTION OF ADVANCED COMPOSITE MATERIAL

Preliminary investigations of advanced composite materials reveals that ACM presents no danger to human health in their post manufactured state.

Combustion and thermal decomposition of ACM appears to cause a potentially toxic chemical transformation. Military aviation mishaps have resulted in illness to fire safety personnel as a suspected consequence of exposure to potentially toxic gases generated from combusted composite materials (Ohlemiller et al., 1993).

In response to mishaps involving ACM components, the Department of Defense (DOD) and the airline industry have been prompted to investigate the potential short and long-term toxic effects of combusted and thermally decomposed composite materials. Additionally, concerns involving human exposure to previously combusted and stored ACM (i.e. off-gassing) have been raised.

Off-gassing of thermally decomposed/combusted ACM and subsequent storage of this material in a confined area is related to compounds evolved during thermal decomposition/combustion as the vapor-phase organics cool below their boiling point.

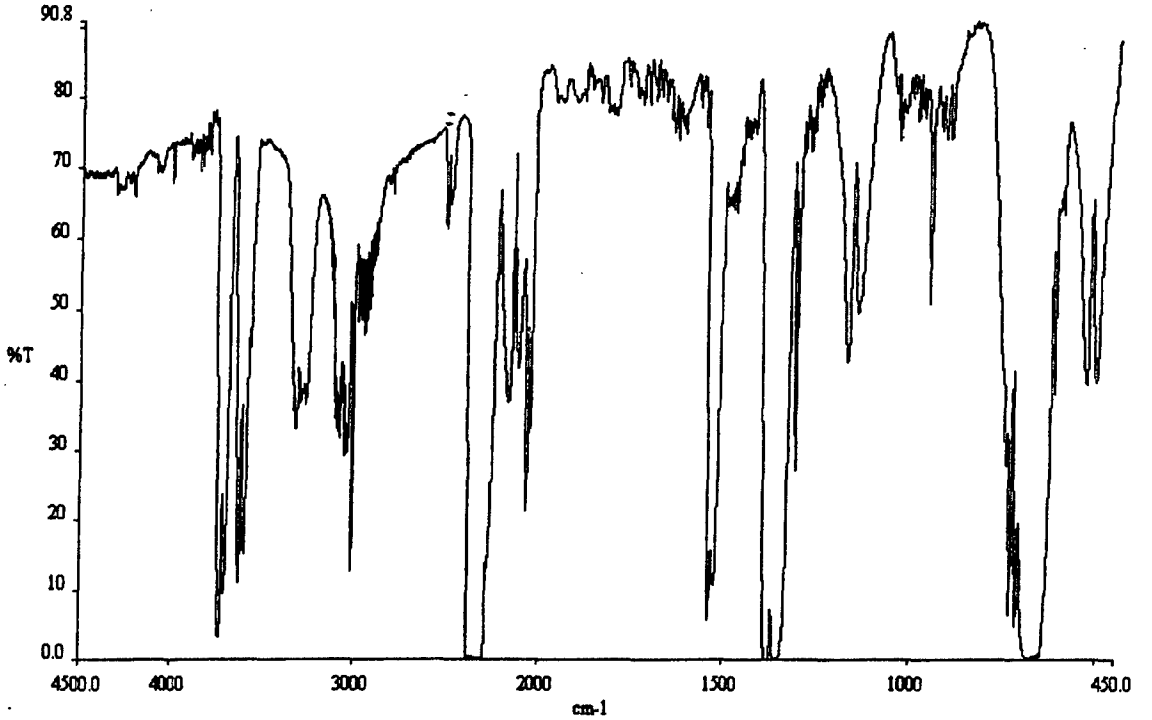

Figure 2. Typical ACM Burn FTIR Spectrum

FIGURE 7.3-2. TYPICAL ACM BURN FTIR SPECTRUM

Short-term toxic effects would come from acute inhalation exposure (respirable particulate and gas-phase organic material) from an ACM fire and sub-chronic exposure via previously combusted (off-gassing) ACM. Gases in the smoke plume of an ACM fire are a complex mixture whose components may interact in a synergistic, additive or antagonistic manner.

Additionally, the mixture produces a convoluted measured IR spectrum (refer to Figure 7.3-2). Thus, principle component regression or partial least squares (Pottel, 1995) should allow the differentiation of chemical compounds in the (IR) spectrum. Preliminary investigation (Courson et al., 1996) indicated the presence of numerous organic compounds (aniline, phenol, quinoline, and others) associated with particles released during the combustion of ACM.

Therefore. a dose-response model must consider a mixed dose (multiple gases and organics) as opposed to a single dose in addition to time of exposure. Since the response data is categorical, the most appropriate statistical model is the logistic regression.

The authors opted to compose the statistical routines in FORTRAN with an IMSL subroutine package. This provides end users access (with minimal training) to the N-gas and fractional effective dose models, previously reserved for individuals with proficiency in manipulating statistical software. SYSTAT version 6.1 and BMDP version 7.0 statistical packages were utilized to testing the FORTRAN model program.

MODEL (PROGRAM) ORGANIZATION

The main program (ACMMODEL) (see Figure 7.3-3) contains a chief menu, directing the user to a particular submenu of the model (fractional effective dose, N-gas, physiological, principal component regression, principal component regression-application and logistic regression). All submenus originate and conclude at the primary menu, in addition to permitting the operator to exit the program. The primary screen presents the administrator with seven menu selections.

The first (1) selection calls the fractional effective dose subroutine (CPSFED). This fractional effective dose calculation, as defined by Hartzell (Hartzell, 1989), can be used to estimate the time to incapacitation for personnel (i.e. firefighters, flightline crews, etc.) exposed to the evolving gases from combustion of ACM. The fractional effective dose requires the numerical integration of the function $(C_i(t) - b_i)/K_i$, where $C_i(t)$ is the toxicant concentration, and K_i and b_i are the slope and the intercept of the linear function of the reciprocal of the time of exposure and LC_{50} of the i^{th} chemical component.

Integration was done using the trapezoidal rule (TRAP). The trapezoidal rule estimates the area under the curve by partitioning the area into trapezoids, calculating the area of each trapezoid, and summing the areas of calculated trapezoids. Subroutine (CPSFED) asks for the number of chemicals, the time interval (in seconds) for each measurement, the name of chemical concentration (ASCII) data file, and the name of the slope and intercept data file. It reads the data from the above files, and calls the fractional effective dose subroutine (SFED), which in turn summons the trapezoidal rule (TRAP) subroutine. The SFED subroutine determines critical time to incapacitation, time stamps the data, in addition to offering the user a print option for collected data.

The second (2) choice is the N-gas model subroutine (NGAS). The N-gas model utilizes the time weighted concentration of seven gases (CO, CO_2, O_2, HCN, HCl, HBr, and NO_2). If a gas concentration is collected in real-time, the time weighted concentration is found by numerical integration using Simpson's rule (SIMPNG). If a gas concentration is not collected in real-time, the subroutine asks for a specific (average) gas concentration.

Should a gas not be present during combustion, the user enters a zero (0). The LC_{50} values for the above gases are supplied by the N-gas subroutine. The user can select option (1) exposure only or a combination of exposure and post exposure scenarios, option (2). The subroutine (NGAS) asks for the number of real-time gases and the

name of the ASCII file which contains the real-time gas data. The slope and the intercept of the line between the concentration of CO_2 and the concentration of CO are included. If NO_2 is missing from the sample and the selection is exposure/post exposure, the subroutine calculates the

$$\text{N-gas value} = \text{slope}*[CO]/([CO_2] - \text{intercept}) + [HCN]/LC_{50}HCN + (21 - [O_2])/(21 - LC_{50}O_2) + [HCl]/LC_{50}HCl + [HBr]/LC_{50}HBr.$$

If NO_2 is missing and the scenario is exposure only, the subroutine calculates the

$$\text{N-gas value} = \text{slope}*[CO]/([CO_2] - \text{intercept}) + [HCN]/LC_{50}HCN + (21 - [O_2])/(21 - LC_{50}O_2).$$

IF NO_2 is present and the selection is exposure only,

$$\text{N-gas value} = \text{slope}*[CO]/([CO_2] - \text{intercept}) + (21 - [O_2])/(21 - LC_{50}O_2) + 0.4*[NO_2]/LC_{50}NO_2*([HCN]/LC_{50}HCN + 1).$$

If NO_2 is present and the preference is exposure/post-exposure,

$$\text{N-gas value} = \text{slope}*[CO]/([CO_2] - \text{intercept}) + (21 - [O_2])/(21 - LC_{50}O_2) + 0.4*[NO_2]/LC_{50}NO_2*([HCN]/LC_{50}HCN + 1) + [HCl]/LC_{50}HCl + [HBr]/LC_{50}HBr.$$

The value in [brackets] in the above formulas is the concentration in ppm*min of the gas (except for O_2, which is percent*min). The N-gas value is then printed. If the N-gas value is greater than 1.3, the value is flagged and printed with the message that the combination of gases will lead to termination.

The third (3) option is the physiological model (currently under development). The physiological model will include fractional effective dose concepts, actual physiological uptake of toxicants, known physiological reactions of humans to the seven gases (CO, CO_2, O_2, HCN, HCl, HBr, and NO_2), and a physiologically based pharmacokinetic model of the top organic compounds. A separate FORTRAN subroutine to address the physiological segment is planned.

Option four (4) is the principal component regression-model development (CPPCR). Smoke, created by combusted ACM, is analyzed using Fourier Transform Infrared Spectroscopy (FTIR). FTIR operation stores the data as ordered pairs of numbers (wavenumber, absorbance). A relationship between absorbance and concentration is expressed by Beer's law, which states that absorbance is concentration times absorptivity times path length.

An IR spectrum is displayed as a graphical plot of the above ordered pairs. Because smoke contains multiple gases that produce interfering spectra, the traditional area of absorbances to concentration calibration curves are not adequate to determine the concentration of various gases in the smoke. The principal component regression finds the fractional part of the total spectrum that a particular gas contributes, utilizing reference spectra obtained from pure compounds and spectra from GC/IR runs of organic compounds collected from the combustion process.

This information will yield a relative concentration for each compound identified. These relative concentrations, when compared with a total quantity of the compound found in the burn (obtained through other analytical methods), will allow the concentration of each compound to be estimated at the time each spectra was taken.

Option 4 takes the experimental infrared data and performs the regression, separating the known components. Users must supply spectra of known gases and organics to the subroutine through an ASCII data file which the subroutine asks for by file name. Subroutine 4 provides the necessary output which is used by selection five.

Option five (5) is the principal components application (FCPC).

The coefficients from selection four relate the concentration of a known mixture of gases to the factor scores (result of mixture separation). The identified mixture (concentration = factor scores x coefficients + error) is applied to a new set of factor scores derived from the spectrum of a smoke sample. The concentration of the gases in the newly acquired sample is estimated by multiplying the new factor scores by the coefficients (from selection four). Concentration of the gases is stored in a file which can be accessed by other sections of the model such as N-gas, fractional effective dose, physiological model and logistic regression.

Choice six (6) is the logistic regression subroutine (LRACM). The dependent variables are pathological results which are: 0 = lesion absent and 1 = lesion present-(quantile). Thus logistic regression was chosen to relate pathological results with concentration, time of exposure and blood gas. Utilization of the logistic regression model may be used to predict the potential for adverse health effects in an exposed individual. To exit the ACM model, select option seven.

```
Program ACMMODEL
C
C    Program ACMMODEL Does ACM Modeling Using The Following:
C    N-GAS Model(Toxic Potency)
C    Fractional Effective Dose(Time to Incapaciation)
C    Physicological Model
C    Principal Component Regression(Estimation of Chemical
C    Concentration)
C    Logistic Regression(Dose-Response and Risk Assessment)
C
C    Subroutines Used
C
     External CPSFED,NGAS,CPPCR,FCPC
C
C    Main Menu to Choose One of the Above Models
C
     Integer TMODEL
  10 Write(*,'(A\)') ' Choose from One of the Following:'
```

Figure 7.3-3. FORTRAN Code for the Main Program-ACMMODEL

```
      Write(*,'(A\)') '   1 = Fractional Effective Dose.'
      Write(*,'(A\)') '   2 = N-GAS Model.'
      Write(*,'(A\)') '   3 = Physicological Model.'
      Write(*,'(A\)') '   4 = Principal Component Regression-Model'
      Write(*,'(A\)') '       Development.'
      Write(*,'(A\)') '   5 = Principal Component Regression-Application'
      Write(*,'(A\)') '   6 = Logistic Regression.'
      Write(*,'(A\)') '   7 = None of the Above.'
      Write(*,'(A\)') ' Enter One of the Above Choices: '
      Read(*,*) TMODEL
      If(TMODEL.EQ.1) Then
        Goto 20
      Else If(TMODEL.EQ.2) Then
        Goto 30
      Else If(TMODEL.EQ.3) Then
        Goto 40
      Else If(TMODEL.EQ.4) Then
        Goto 50
      Else If(TMODEL.EQ.5) Then
        Goto 60
      Else If(TMODEL.EQ.6) Then
        Goto 70
      Else
        Goto 80
      End If
C
C     Fractional Effective Dose Section
C
   20 Call CPSFED
      Goto 10
C
C     N-GAS Model Section
C
   30 Call NGAS
      Goto 10
C
C     Physiological Part of the Model
C
   40 Call PHYSMOD
      Goto 10
C
C     Principal Component Regression Section Which Will Deal With
C     Interference Problems in Spectrum
C
   50 Call CPPCR
      Goto 10
C
C     Principal Component-Application
C     Finds the Gas Concentration from Interfering Spectrum in the
C     Smoke.
C
   60 Call FCPC
      Goto 10
```

Figure 7.3-3. (Cont.) FORTRAN Code for the Main Program-ACMMODEL

```
C
C    Logistic Regression
C    Used to Predict the Long-Term Effects of an Exposure to Combustion
C    By-Products.
C
  70 Call LRACM
     Goto 10
C
C    End of Program
C
  80 Stop
     End
```

Figure 7.3-3. (Cont.) FORTRAN Code for the Main Program-ACMMODEL

CONCLUSION

The Advanced Combustion Model is modular for several reasons. 1) Ease of module integration. 2) Individual components are readily upgradeable/adaptable. 3) Modules can act independently of other model components. 4) Integration of statistical components (principal component regression and logistic regression) are no longer dependent upon a statistical software package for manipulation. 5) Utilization of FORTRAN code allows the model to be functional on a computer which has either a FORTRAN compiler or a Windows operating platform (it currently operates utilizing the MS-DOS component of Windows).

Eventually, the ACM combustion model will be developed to function fully under the Windows 95 operating platform. Adaptation of the ACM combustion model can potentially involve future combustion related toxicity studies.

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SECTION 8

METHODS DEVELOPMENT

8.1 PHARMACOKINETICS OF DERMAL ABSORPTION IN RODENTS WITH SMALL ORGANIC CHEMICALS

C.M. Garrett and J.N. McDougal

ABSTRACT

Understanding dermal absorption of organic chemicals is very important because of their ubiquitous nature in the workplace, home, and environment. If we want to be able to adequately assess human health hazards from these chemicals, we need to be able to predict the amount of chemical which might be absorbed into the body in a wide variety of potential exposure situations. We can predict hazard with a fair degree of certainty only when we understand the chemical, biochemical, physiological, and anatomical characteristics which affect dermal absorption. Carefully controlled experiments using laboratory animals as surrogates for humans is the best way to gain the understanding necessary to extrapolate to humans. Our goal is to be able to extrapolate from rodents to humans based on a solid understanding of the anatomical differences. We have chosen three rodent species (guinea pig, hairless guinea pig, and rat) which have previously measured differences in such things as skin thickness, hair follicle density, and capillary depth to do absorption studies. Three chemicals (perfluorohexyl iodide, PFHI; chloropentafluorobenzene, CPFB; and dichlorobenzene, DCB) which each have different lipid solubility were also chosen. We have investigated the dermal absorption of these chemicals in each species during a four hour period by analyzing blood concentrations during a carefully controlled exposure. We report blood concentrations for the studies which have been completed to date. CPFB exposures produced the highest blood concentrations, followed by DCB and PFHI. Blood concentrations (0.02 to 0.06 μg/mL) after four hours exposure to PFHI were similar for each of the three species. Four-h CPFB blood concentrations ranged from 0.5 to 5.5 μg/mL. Four-h blood concentrations in the rat were about five times as high as in the guinea pig and the hairless guinea pig. Final blood concentrations for DCB ranged from 1 to 2 μg/mL in the guinea pig and rat (hairless guinea pig not completed yet). Rats tended to have the highest blood concentrations when there were species differences. The reason for this may be that rats have the thinnest epidermis and stratum corneum. They are also smaller than the guinea pigs and therefore the same exposure (surface area and concentration) would be expected to provide the highest blood concentration. Absorption parameters (flux and permeability coefficients) will be determined in the future using physiologically-based pharmacokinetic (PBPK) models. The analysis with PBPK models will be necessary to determine if the differences are due to anatomical differences in the skin or differences in body weight.

INTRODUCTION

The integumentary system is the largest organ system in the body and serves as a protective barrier against many environmental stresses. Historically, it was thought that this barrier was complete and impenetrable. It is now

known however, that many substances are able to penetrate the skin in limited amounts at rates corresponding to their lipid solubility (Scheuplein, 1983). Anatomical differences within body sites and between experimental species (primarily rodents) and humans are also recognized to affect dermal absorption rates. The skin has three major layers which may affect the penetration of chemicals: the epidermis, the dermis, and the subcutaneous tissues (hypodermis). The deepest skin layer, the hypodermis, consists of adipose tissues, connective tissues and smooth muscle. Directly above the hypodermis lies the dermis which is vascular and contains glands and hair follicles. The epidermis is the tightly packed outermost layer of skin which is primarily responsible for the barrier function of the skin (Montiero-Riviere, 1996). The barrier property of the epidermis is due largely to the outermost layer; the stratum corneum. The stratum corneum is composed of tightly adherent keratinized squamous cells enriched with intercellular lipids (Downing et al., 1987). It is the lipoidal nature of the stratum corneum that provides a barrier to water loss, but may allow the penetration of many lipid soluble chemicals.

Anatomical structures in the skin such as hair follicles and glandular appendages can also influence penetration of chemicals, although the primary route of penetration seems to be through the general skin surface and not these appendages. There is often significant anatomical variation in skin between species and within individuals of the same species. For example, the area represented by follicles and glands in humans is relatively small. So for humans at least, hairy skin may be no more permeable to chemicals than non-hairy skin (Leung, 1994). On the other hand, for rodent skin, hair appeared to contribute significantly to overall percutaneous absorption (Kao et al., 1988). We previously measured the number of hair follicles per square millimeter in the three laboratory strains by image analysis (Grabau et al., 1994). A surprising finding from this investigation was that the hairless guinea pigs had more follicles than Hartley guinea pigs, but the hairless guinea pig follicles contain immature hairs. Rats had more follicles per unit area than either guinea pig strain (Figure 8.1-1).

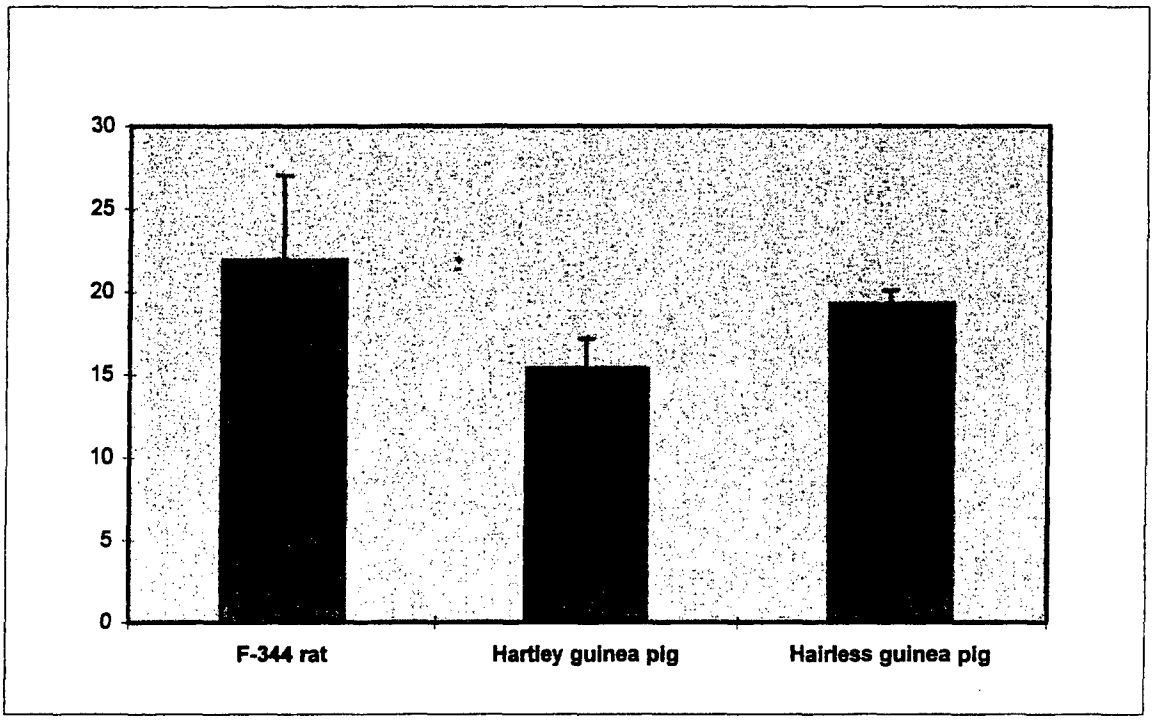

Figure 8.1-1. Number of hair follicles/mm^2 in the three laboratory strains.

Therefore, it is important to consider these species differences when predicting human responses from animal data. Another variable which affects overall penetration and differs among species is the thickness of the epidermis (Scheuplein, 1983). Since the epidermis represents the rate limiting barrier to dermal penetration, we would expect that a species with a thick epidermis will exhibit lower overall percutaneous absorption than a species with a thin epidermal layer. Fischer 344 (F-344) rats, Hartley guinea pigs, and hairless guinea pigs were selected for this study because of measured differences in skin anatomy (Grabau et al., 1994). Of the species tested, hairless guinea pigs have the thickest stratum corneum and F-344 rats have the thinnest (Figure 8.1-2.).

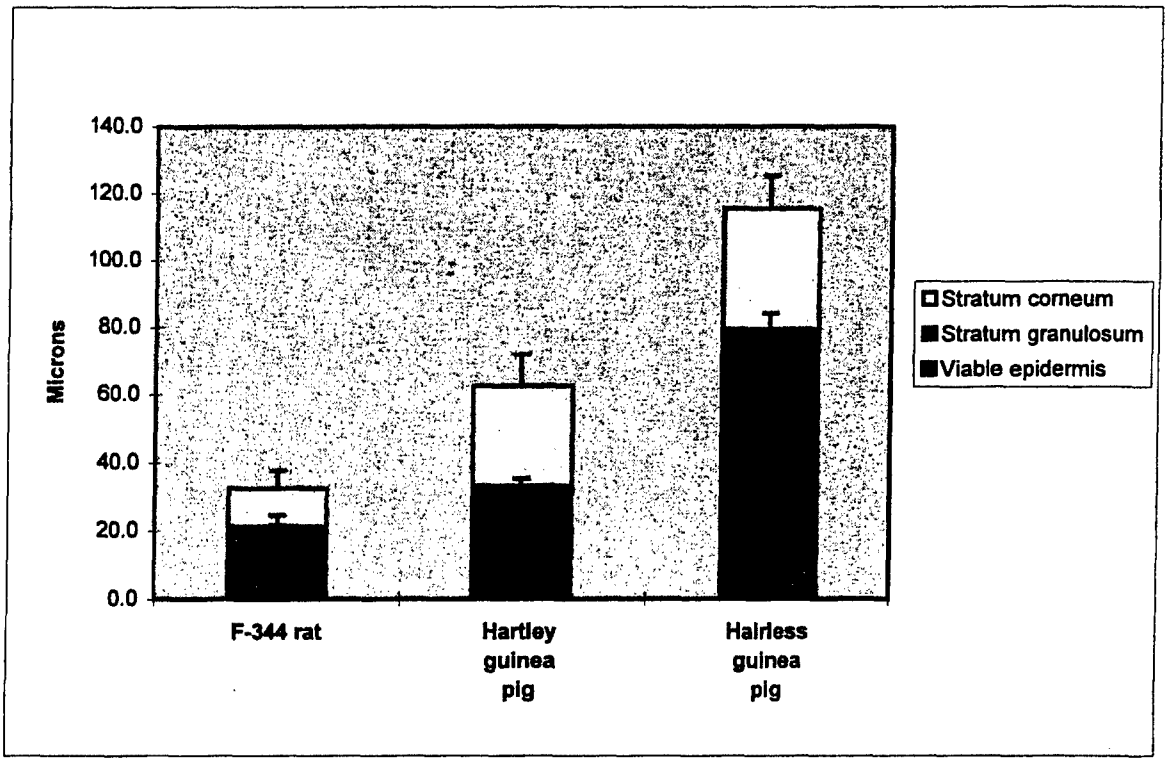

Figure 8.1-2. Differences in thickness of epidermal components of three laboratory species.

Depth of dermal capillaries can also influence dermal absorption of chemicals. The hypothesis is that a chemical will have to diffuse farther to reach the bloodstream of an animal with very deep capillaries. Capillary depth was measured from the outermost surface of the skin to the geometric center of the vessel. Rats have the deepest capillaries (593 μm), followed by Hartley guinea pig (495 μm) then hairless guinea pig (471 μm) (Figure 8.1-3.). The thickness of the dermis may affect the absorption process by providing a reservoir for chemical deeper in the skin than the capillary loops. Species related differences in dermal thickness were also measured by image analysis. F-344 rats have the thinnest dermis followed by hairless guinea pigs. Hartley guinea pigs have the thickest dermis of the species measured (Figure 8.1-4.).

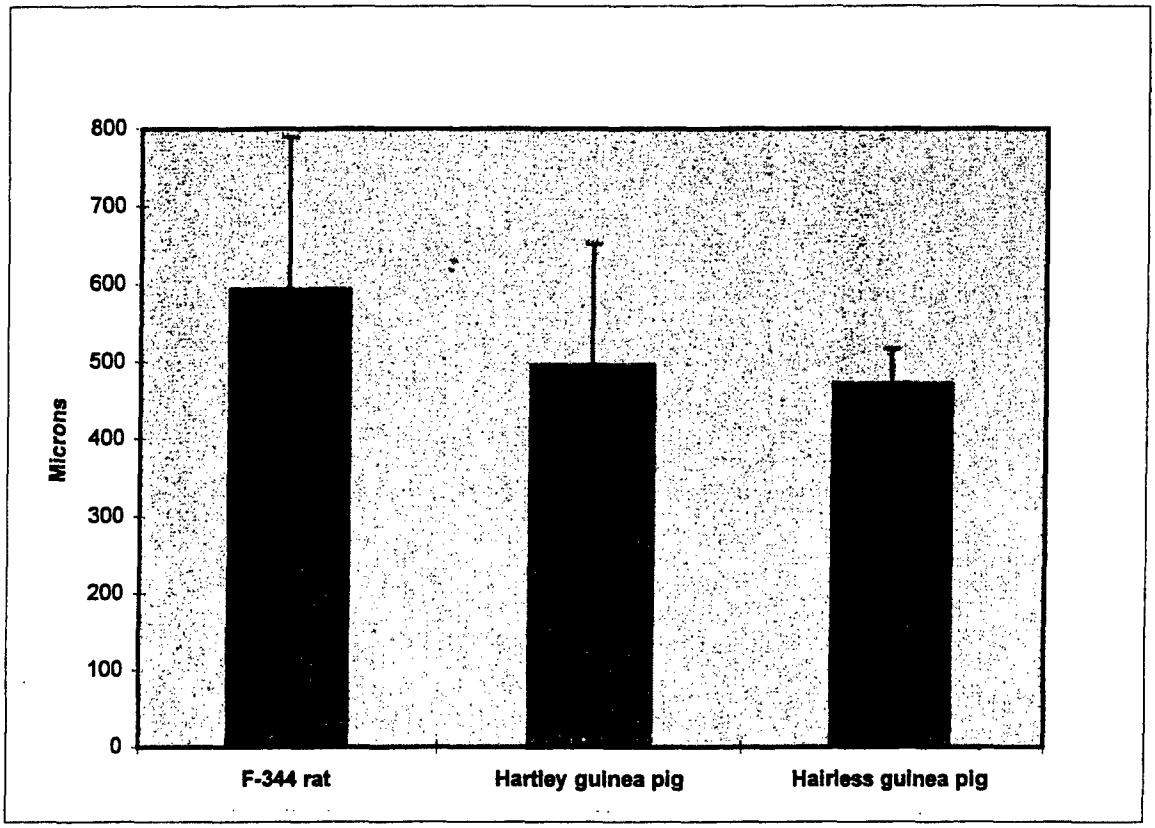

Figure 8.1-3. Depth of dermal capillaries as measured by image analysis.

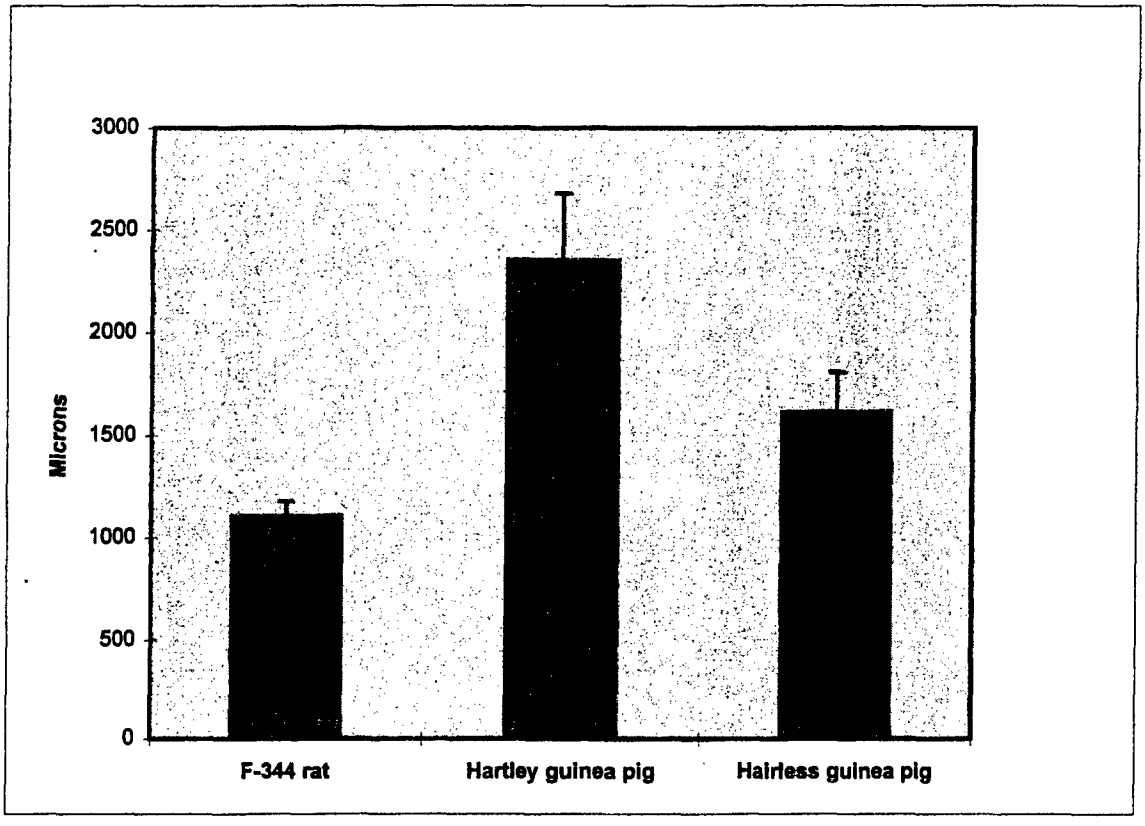

Figure 8.1-4. Species related differences in dermal thickness measurements.

We used three test chemicals; Perfluorohexyliodide (PFHI), Chloropentafluorobenzene (CPFB), and Dichlorobenzene (DCB), to investigate whether the impact of anatomical characteristics are chemical category specific. Table 8.1-1 shows the physical characteristics of these chemicals. All chemicals have six carbons, but vapor pressure varies by a factor of 30, molecular weight differs by a factor of three and fat/air partition coefficient are different by more than two orders of magnitude. In general, small molecular weight chemicals penetrate better than large molecular weight chemicals and lipophilic chemicals are absorbed into the skin more readily than chemicals which are not lipophilic (Flynn, 1990). It is possible that chemicals which penetrate the skin most readily have moderate lipid solubility and moderate water solubility.

TABLE 8.1-1. Physical characteristics of the chemicals used. Partition coefficients were measured in the laboratory using a modified vial equilibration method.

Chemical	Vapor Pressure (mmHg)	Molecular Weight	Water Solubility	Fat/Air Partition Coefficient
DCB	1.0 @ 20°C	147	fairly insoluble	26,606
CPFB	14.1 @ 25°C	202	practically insoluble	766
PFHI	29.6 @25 °C	446	insoluble	131

The purpose of these studies was to measure blood concentrations during the absorption of three model chemicals in three carefully chosen rodent strains. Results from this study can be used to determine absorption parameters (flux and permeability coefficients) using physiologically-based pharmacokinetic (PBPK) models. An attempt will be made to correlate absorption parameters in each species with anatomical characteristics and determine if the physical characteristics chemicals affect the correlations.

MATERIALS and METHODS

Animals and Chemicals

Fischer 344 rats (200-250 g), Hartley Guinea Pigs (300-350 g), and hairless Guinea Pigs (300-350 g) were obtained from Charles Rivers Laboratories. Perfluorohexyl Iodide, Chloropentafluorobenzene, and Dichlorobenzene were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Pre-exposure Preparations

Animals were given an intra-peritoneal injection of an anesthetic solution (70 mg/kg Ketamine HCl and 6 mg/kg Rompun) at a dose of 1 mL/kg body weight. Once the animal was properly anesthetized, it's back, the underside of it's neck and the upper shoulder area were closely clipped with electric clippers. The surgical site was cleaned with Betadyne solution and wiped with isopropyl alcohol. Surgeries were performed under aseptic conditions in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals".

A small incision, approximately 2.0 cm in length, was made from just below the jaw line to just over the right clavicle. The right external jugular vein was exposed by blunt dissection, and a silastic tubing cannula was inserted into the vein. Proper flow of blood through the cannula was ensured by drawing back on a 1 cc syringe attached to the end of the cannula. Next, the cannula was tunneled beneath the skin and exteriorized at the back of the neck where it was sutured to the skin at the dorsal site. Two-to-three stainless steel wound clips were used to close the initial incision. The cannula was flushed with 0.1 mL of heparinized saline (5% heparin and 95% 0.9 normal saline) to prevent the formation of blood clots and was kept in place by using a modified harness made out of 2.0 in-wide Vetrap (3M St. Paul, MN). Finally, an exposure cell was affixed to the closely clipped mid-dorsal region of the animal with Zap-a-Gap superglue. Care was taken to ensure a tight seal between the glass exposure cell and the skin of the animal.

Exposures

Four-h exposures were conducted on the day following surgery. During exposure the animals were housed singly in a ventilated cage rack with independent air flow to each cage. This assured that a leak from the cell of one animal would not allow the other animals to breathe the vapors. Two milliliters of chemical was injected through the septum of the exposure cell. A 0.2 mL blood sample was taken from each animal at 0, 15, 30, 45, 60, 120, and 240 min after dosing. Blood volume was replaced with heparinized saline. Samples were equilibrated at 60 °C for 90 min with gentle shaking. Headspace concentrations were analyzed using an external standard curve.

Gas Chromatography

A Hewlett Packard 5890 gas chromatograph equipped with an electron capture detector and a Hewlett Packard 19395A headspace autosampler was used for the analysis of blood samples from the exposures. Samples in the auto-injector were kept at 60 °C; sample loop temperature was 65 °C. A 1.0 mL aliquot of headspace was injected onto a DB-1 column.

Conditions for PFHI and CPFB Analysis

Oven temperature was 90 °C. The injector temperature was 150 °C and the detector temperature was 300 °C. A carrier gas of 95% argon and 5% methane was used. Flow through the column was 5 mL/min with a total flow of 25 mL/min.

Conditions for DCB Analysis

Oven temperature was 150 °C. The injector temperature was 175 °C and the detector temperature was 300 °C. A carrier gas of 95% argon and 5% methane was used. Flow through the column was 5 mL/min with a total flow of 25 mL/min.

RESULTS and DISCUSSION

The exposures with PFHI show a linear increase in blood concentration up to 4 h after an initial period of slower absorption of approximately 1 h (Figure 8.1-5.). At 4 h, blood concentrations were 0.03 μg/mL in hairless guinea pigs, 0.04 μg/mL in Hartley guinea pigs, and 0.07 μg/mL in Fischer 344 rats. No statistical difference in blood concentration was found between the animal groups.

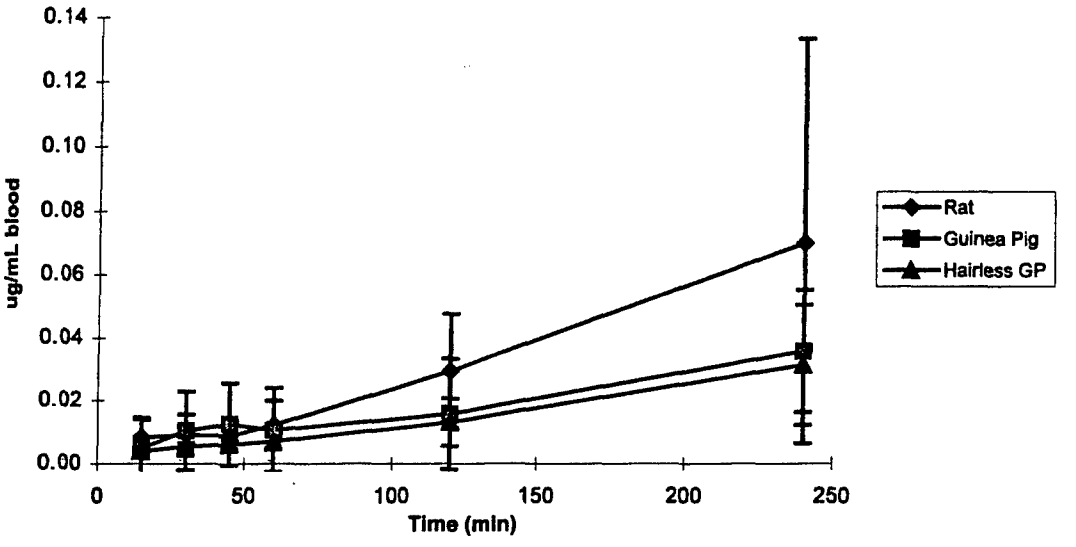

Figure 8.1-5. ***In vivo*** **dermal exposures of three laboratory species to Perfluorohexyl iodide.**

After a 4-h dermal exposure to CPFB, mean blood levels were 0.6 μg/mL, 1.4 μg/mL, and 5.6 μg/mL for Hartley guinea pigs, hairless guinea pigs, and F-344 rats, respectively (Figure 8.1-6.). Blood concentrations were higher in rats than either of the guinea pig strains.

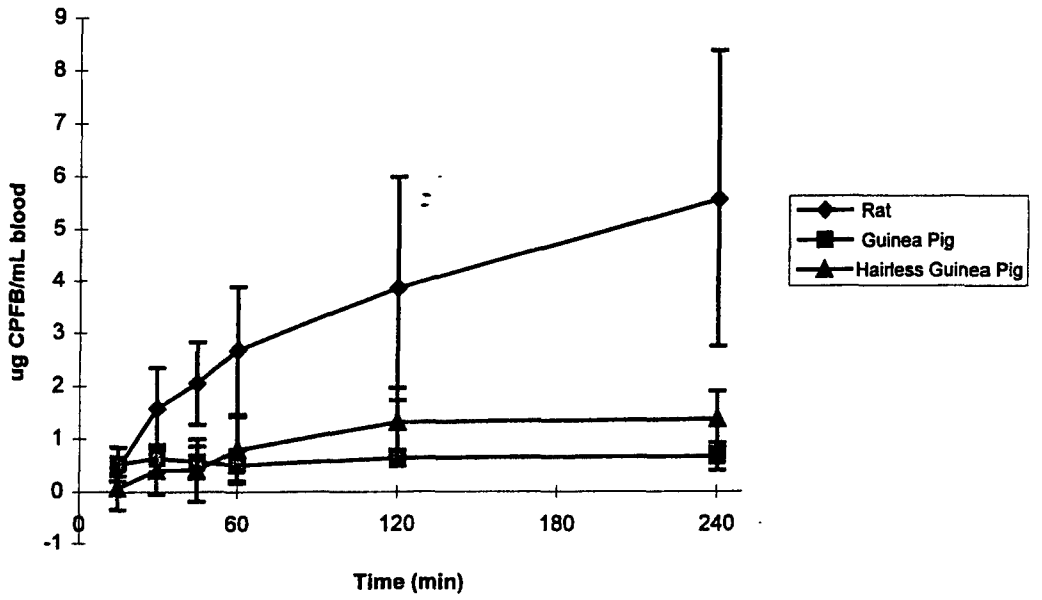

Figure 8.1-6. ***In vivo*** **dermal exposure to CPFB in rats, Hartley and hairless guinea pigs.**

Four-h mean DCB concentrations were 1.2 μg/mL, and 2.0 μg/mL for Hartley guinea pigs and rats, respectively (Figure 8.1-7.). Exposure to DCB has not yet been conducted in hairless guinea pig. DCB concentrations in rat blood rise linearly up to 1 h, then level off. In the guinea pig, initial DCB blood concentrations are high then decrease at 1 h and remain level for the duration of the exposure. No statistical difference in blood concentration was found between the animal groups.

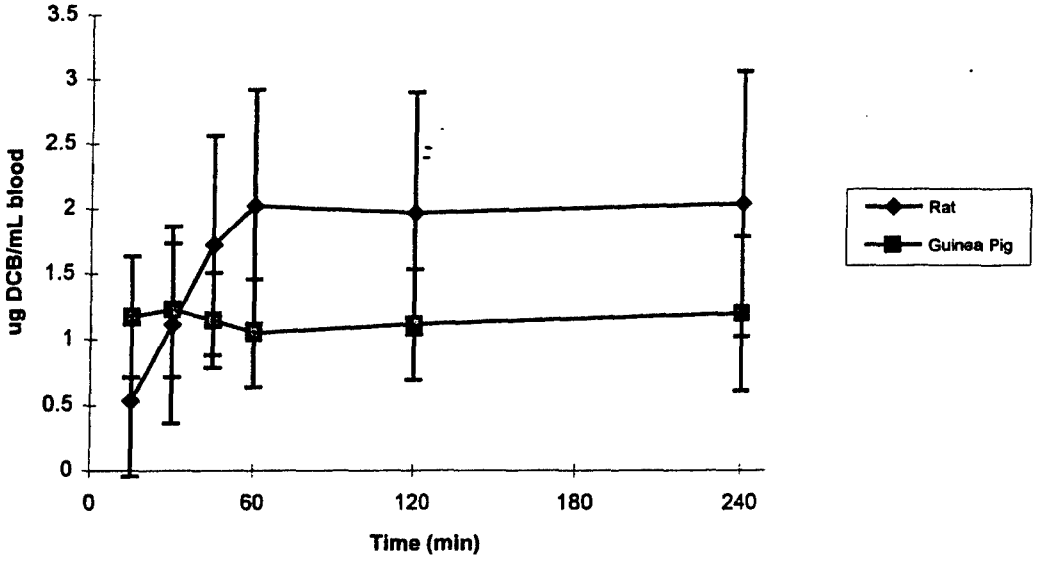

Figure 8.1-7. ***In vivo*** **dermal exposure to DCB in rats and guinea pigs.**

In all compounds tested, rats had a tendency for higher blood concentrations of the chemical than either strain of guinea pig, but this was not significant with PFHI. This could be due to the fact that PFHI does not penetrate the skin very well. Species differences are more pronounced with better penetrating chemicals such as CPFB and DCB (Figures 8.1-6. and 8.1-7.). Body weight (and, therefore, blood volume) impact the concentration of chemical in the blood. The rats used in this study were about 100 grams smaller on average than the hairless guinea pigs or Hartley guinea pigs and, therefore, have a smaller overall blood volume which could indicate a more concentrated sample. Each of the exposure areas was identical and the percent of surface area exposed would decrease as the body weight increases.

It appears that the shape of the blood concentration vs. time curves may be different between species. In all exposures conducted with guinea pigs there was a slight initial rise in blood concentration followed by a slight decline before the concentrations level off. CPFB and DCB exposures in rats also show an initial rise in blood concentration but in the guinea pig this rise was followed by a decrease in concentration at 60 min which was not apparent in the other strains. The significance of this (if any) is not known.

At the end of these exposures, blood concentrations were significantly higher in rat, than in either strain of guinea pig (Figures 8.1-5, 8.1-6, and 8.1-7). This also correlates well with the measurements of epidermal thickness (Figure 8.1-1a.). As expected, percutaneous absorption seems to increase with decreases in thickness of epidermal components. On the contrary, the measured values for capillary depth do not seem to correspond to the blood concentrations from *in vivo* exposures (Figures 8.1-3, 8.1-5, and 8.1-7). It was expected that the

species with the most superficial capillaries, hairless guinea pig, would show the greatest levels of chemical in the blood, however the results do not support this hypothesis. We measured average capillary depth which may include the deeper capillaries that don't contribute to the entry of the chemical into the general circulation. The rat with its deeply penetrating follicles causes the average to be deeper than the other species. In rodent species, however, the presence of hair may contribute to the overall penetration of chemical through the skin (Kao, 1988).

Hence the number of hair follicles present in the exposed area could contribute to the total blood concentration. Number of follicles corresponds to *in vivo* blood concentrations in all but hairless guinea pig species. In this case, there are a larger number of follicles despite the lack of development of the hair shaft itself (Figure 8.1-1). This finding reinforces the function of hair in dermal penetration.

CONCLUSIONS

These *in vivo* exposures are useful to compare species differences in blood concentrations during dermal exposures. CPFB exposures resulted in the highest blood concentrations, followed by DCB and PFHI. As expected, blood concentrations of Perfluorohexyl iodide were very low in each of the three species tested. However, species differences in penetration are evident in two of the three chemicals tested in this study. The ranking of the blood concentrations may not correlate with the ranking of the absorption parameters which will be determined using PBPK models in the future.

ACKNOWLEDGMENTS

The authors gratefully acknowledge funding by the Air Force Office of Scientific Research (AFOSR/NL). The animals used in these studies were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals Resources, National Research Council, DHHS, National Institutes of Health Publication #86-23, 1985, and the Animal Welfare Act of 1966, as amended.

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8.2 ESTABLISHMENT OF A BRAIN SLICE TECHNIQUE AS PART OF THE *IN VITRO* ASSESSMENT SYSTEM FOR SCREENING NEUROTOXICANTS

J. Lin, J. Rossi III[1], and A.F. Nordholm[1]

ABSTRACT

Development of a highly reliable, rapidly deployable *in vitro* cellular-molecular level assessment system for inclusion in the Navy Neurobehavior Toxicology Assessment System (NTAS) to be used for risk assessment and treatment of exposure to neurotoxicants is one of the primary interests of the neurobehavioral group at NMRI/TD. The *in vitro* brain slice preparation technique, which provides investigators with many was developed to investigate the cellular and molecular alterations induced by certain neurotoxicants, chemicals or drugs. The current work efforts have shown that a healthy brain slice preparation and a stable intracellular electrophysiological recording system have been successfully developed at NMRI/TD. As a continuation of the previous studies and to validate the newly established system, the actions of trimethylolpropane phosphate (TMPP), a potent convulsant, were tested on hippocampal CA1 pyramidal cells using intracellular electrophysiological methods *in vitro*.

INTRODUCTION

Navy and Marine Corps personnel are commonly exposed to a diversity of interacting chemical toxicants in the workplace and in combat deployments. Examples of these exposures include: jet fuels, gasoline and kerosene, paints, solvents, munitions, pyrolysis products, and ozone-depleting substance replacements. While some exposures occur at high concentrations and result in immediate lethality or performance incapacitation, the majority occur repeatedly at dose levels inadequate to induce physiological irritancy or observable decrements in ongoing performance. As has been shown in studies of chemical kindling, however, the consequences of repeated exposure to low doses of certain chemical toxicants can include manifestation of increasingly more severe behavioral responses to the previously sub-threshold doses (sensitization). Such exposures may induce transient or persisting changes in the structure or functioning of central nervous system (CNS) cells at the cellular and/or molecular level. However, changes at the cellular or molecular level may (at least initially) be sufficiently subtle to avoid detection by the gross behavioral testing comprising the NTAB/GASH (Navy Neuro-Behavioral Toxicity Assessment Battery / Global Assessment System for Humans) neurobehavioral batteries. Investigation, treatment, and prophylaxis of syndromes involving those toxicants may depend upon development and utilization of cellular-molecular testing methodologies which is part of the NTAS. Our goal is to

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develop and validate a highly reliable, rapidly deployable *in vitro* assessment system for inclusion in the NTAS, and for risk assessment and treatment of neurotoxicants of military interest.

Slice preparations provide investigators with several technical advantages. First, thin slices improve visualization of the tissue which makes it possible for an investigator to guide visually both recording and stimulating electrodes to the desired sites and to distinguish cellular regions from fiber tracts and somatic from dendritic regions. One may then impale selectively cell soma or dendrites with recording electrodes and stimulate discrete synaptic or antidromic pathways. Second, slice preparations provide relatively simplified yet maintained neural organization. In removing the tissue from the rest of the brain, the tissue is no longer subject to neurohumoral effects or to inputs from distant brain regions. Unlike cell-culture techniques in which the normal complex organization is lost or drastically altered, the slice preparation maintains the normal complex organization of the brain. Third, the slice preparations eliminate the interference of heartbeat and respiration on recording. Moreover, there is no blood pressure to monitor, no heart rate to stabilize and no respiration to maintain. Fourth, these *in vitro* preparations will allow an investigator to accurately deliver toxicants, drugs and even putative transmitter substances directly to the specific site or brain region with a known concentration without worrying about the blood brain barrier effect. The extracellular environment, which is essential for cell spiking activity, for synaptic release of transmitters and for mediation of postsynaptic potentials, can be accurately controlled and easily altered.

TMPP, a potent neurotoxicant generated from the pyrolysis of certain phosphate ester-based lubricants or fire-retarded polyurethane foams, was tested in the present study. To validate the established testing system, the action of TMPP was studied on hippocampal brain slices by using intracellular electrophysiological methods.

EXPERIMENTAL DESIGN AND METHODS

Brain Slice Preparation

Experiments were performed on male Sprague-Dawley (Crl:CD^{R}) rats weighing 120-250 g. After rats were guillotined, the brains were rapidly removed and cooled in 4 °C artificial cerebrospinal fluid (ACSF), composed of (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$ and 10 Dextrose and was saturated with 95% O_2 and 5% CO_2 (pH 7.4). Transverse slices (400-500 µm thick) were prepared from one hippocampus. One slice was held submerged between two nylon nets in the constant perfusion experimental chamber at 30-34 °C, while the other slices were kept in an incubation chamber at room temperature (22-24 °C). Slices were allowed to recover for 1-2 h before any electrophysiological recordings were performed.

Electrophysiology

Standard electrophysiological techniques for stimulation and intracellular recording were used. Intracellular electrodes were filled with 3M KCl and will be filled with 2M potassium methylsulfate (KCH_3SO_4) or 4M potassium acetate (KOAc) in future experiments. The tip resistance of microelectrodes ranged from

50-85 MΩ. Membrane potential was recorded by conventional microelectrode techniques. An active bridge circuit allowed current injection into neurons through a recording microelectrode. Fast excitatory postsynaptic potentials (fEPSPs) and associated action potentials were evoked by electrical stimulation of afferent fibers in stratum radiatum.

Chemicals and Drugs

In the current study, drugs were applied to the cell by either pressure ejection through a picospritzer or superfusion in the medium. TMPP (4-ethyl-1-phospha-2,6,7-trioxa-bicyclo-[2.2.2]-octane-1-oxide) (synthesized by Dr. Paul Servé, Department of Chemistry, Wright State University, Dayton, OH) and γ-aminobutyric acid A ($GABA_A$) receptor agonist Muscimol hydrobromide (Research Biomedicals International, Natick, MA) were dissolved in ACSF. Diazepam (Research Biomedicals International, Natick, MA) was dissolved in 100% DMSO for stock solutions. However, the highest concentration of DMSO used in theses experiments was 0.002% and no electrophysiological effects of DMSO (0.001-0.1%) were seen in control experiments.

Data Acquisition and Analysis

Signals from the recording electrode was displayed on an oscilloscope and a computer and recorded on a chart recorder and stored on a digital data recorder. p-CLAMP software was used for data acquisition and analysis throughout the whole experiment period. To help analyze the effects of the toxicants on the health status, function and response of the neurons or tissues, a number of electrophysiological end points were considered. These include changes in passive and active cell membrane properties of neuronal cells being evaluated, changes from baseline in spontaneous and evoked discharges, changes in ion channel activities and changes in synaptic potentials evoked by regular or tetanus (related to neuronal "learning" mechanisms) stimulation including long term potentiation (LTP) and long term depression (LTD).

RESULTS

CA1 pyramidal neurons tested had a mean resting transmembrane potential of -67±1.5 mV (n=21), mean action potential amplitude of 74±2.2 mV (n=21) and mean cell input resistance of 34±15 MΩ (n=21). Eighty percent of neurons tested exhibited spontaneous activities (0.2-4.5 Hz). Spontaneous membrane oscillations (fast and slow EPSPs) were also observed in 38% (8 of 21) of neurons tested.

The actions of TMPP, a potent convulsant, were tested on hippocampal CA1 pyramidal cells using intracellular electrophysiological methods *in vitro*. Bath application of TMPP (1-100 μM) caused membrane hyperpolarization (7±2 mV, n=7) associated with an increase in input resistance (51±8%, n=7) in 41% (7 of 17) of neurons tested and membrane depolarization (3.7±0.5 mV, n=8) associated with an increase in input resistance (32±3%, n=8) in 47% (8 of 17) of neurons tested. TMPP (1-100 μM) increased the spontaneous activities (101±23% increase,

n=9) in 53% (9 of 17) of neurons tested and decreased spontaneous activities (56±8% decrease, n=8) in 47% (8 of 17) of neurons tested. The increases in spontaneous activities induced by TMPP were blocked by muscimol (1-10 μM), a $GABA_A$ receptor agonist (n=4) and diazepam(1-10 μM), a GABA-benzodiazepine receptor agonist (n=4), respectively. Bath application of muscimol (1-10 μM) or diazepam (1-10 μM) abolished the spontaneous activities of CA1 neurons (n=2 each). TMPP (10 μM) reversed the actions of muscimol and diazepam. Bath application of TMPP (1-10 μM) also induced short epileptiform burst and after spike hyperpolarization (ASH) in 35% (6 of 17) of neurons tested. The amplitude and duration of the ASH are 5-18 mV and 75-300 ms, respectively. Muscimol (10 μM) and diazepam (10 μM) abolished TMPP induced burst and the followed ASH. TMPP did not alter passive or active membrane properties of the CA1 neurons indicating that TMPP has no effect on the sodium and calcium components of action potentials and has no direct toxic effects on the CA1 pyramidal neurons.

DISCUSSION

The data from the present study suggest that neurons in the hippocampal slice preparation exhibited normal transmembrane potentials, input resistance and action potential amplitudes indicating a healthy brain slice preparation. Long term (4-5 h) intracellular recordings from hippocampal neurons have also been successfully established. With this technology, the action of other toxicants of interest, such as HFA-134a, HFC-227ea, Halon 1301, and other ozone-depleting substance replacements, can be investigated at the cellular level.

Data from this study suggest that TMPP induced membrane hyperpolarization as well as membrane depolarization of hippocampal CA1 neurons. The membrane hyperpolarization and depolarization were associated with an increase in input resistance. TMPP increased as well as decreased the spontaneous activities of neurons. The actions of TMPP may be mediated by acting on $GABA_A$-benzodiazepine receptor because the $GABA_A$ agonist muscimol and GABA-benzodiazepine agonist diazepam blocked the TMPP effects and vise versa. In summary, TMPP has excitatory and inhibitory actions on CA1 pyramidal cells. The actions of TMPP may be due to its interaction with $GABA_A$-benzodiazepine receptors. More detailed studies about the actions of TMPP on hippocampal neurons are in progress.

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SECTION 9

CONFERENCE SUPPORT

9.1 1997 TRI-SERVICE TOXICOLOGY CONFERENCE ON ISSUES AND APPLICATIONS IN TOXICOLOGY AND RISK ASSESSMENT

L.A. Doncaster

The Conference on Issues and Applications in Toxicology and Risk Assessment was held at the Hope Hotel and Conference Center, Wright-Patterson Air Force Base, OH, from 7 through 10 April 1997. The conference was sponsored by Tri-Service Toxicology, Wright-Patterson Air Force Base; the Office of Research and Development, United States Environmental Protection Agency; and the Division of Toxicology, Agency for Toxic Substances and Disease Registry. Coordination was handled by ManTech Environmental Technology, Inc., Toxic Hazards Research.

The goals of the conference were (1) exploration of new methodologies for combustion, GI tract, and developmental toxicology; (2) application of guidelines in the risk assessment process; and (3) examination of the issues and approaches for ecotoxicology and pollution prevention.

The 1997 conference was dedicated to Dr. Anthony A. Thomas, the first Director of the Toxic Hazards Division of Armstrong Laboratory. Dr. Thomas died in Arizona on 19 May 1996. He was the designer of the unique "Thomas Domes" for the toxic hazards research facility, which he established from 1964 to 1970, and he was awarded the U.S. patent rights to the special test chambers. Dr. Thomas developed the philosophy and research approach to solve toxicological problems in space cabins and confined spaces, and introduced criteria for emergency tolerance limits for short-term exposure to contaminants. Results of the research have been used to set up emergency exposure limits for toxic chemicals which are applicable to the national space effort, the United States Air Force, and to air pollution problems. Dr. Thomas made significant contributions both to the Air Force and the nation by developing and managing a unique facility for environmental inhalation toxicology research.

The 1997 conference was the first in the series of toxicology conferences to offer concurrent sessions. Sessions 2, 3, and 4 were divided into "a" and "b" sessions and attendees were offered the opportunity to select the subject area most relevant to their interests.

Session 1 of the conference dealt with site-specific risk assessment and included presentations on an overview of the site-specific risk assessment in Superfund (D.A. Bennett); Lavaca Bay: a Superfund case study (J.D. Rauscher); and new developments (A. Guiseppi-Elie). A panel discussion on these subjects followed the presentations.

The subject of Session 2a was combustion toxicology. Included were discussions on new approaches to toxicology - a seven-gas predictive model and toxicant suppressants (B.C. Levin); combustion products from advanced

composite materials (J.C. Lipscomb); pulmonary toxicity of co-exposure to acrolein and aerosol particles in F-344 rats - II (E.C. Kimmel); and chemical and toxicological evaluation of pyrotechnically disseminated terepthalic acid smoke (W.T. Muse, Jr.).

Session 2b covered ecotoxicology and aquatic toxicology and included discussions on Department of Defense activities in ecological risk assessment (J.E. Whaley); activities of the Tri-Service Ecological Risk Assessment Work Group (R.C. Porter); ecological risk assessment guidance for the Superfund Program (D.W. Charters); coordinating activities between the National Oceanic and Atmospheric Administration (NOAA) and other agencies (M.F. Buchman); arctic marine monitoring and assessment - an interagency ecosystems approach (T. Rowles); human exposure to PCBs - modeling and assessment (A. Kinney); and assessing the ecological risk posed by contaminated sediment (T.S. Bridges).

The subject of Session 3a was GI tract toxicity issues and included discussions on acrylonitrile presystemic metabolism and toxicity (A.E. Ahmed); first-pass effect - effect of gastrointestinal metabolism (K.S. Pang); gastrointestinal absorption of metals (G.L. Diamond); and the effect of vehicle, route, and pattern of exposure on the absorption and hepatotoxicity of carbon tetrachloride (J.V. Bruckner).

Discussions in Session 3b dealt with indirect/multipathway exposure assessment and included advances in EPA's indirect exposure modeling methodology - human exposure scenarios (E. Brady-Roberts); the utility of screening models for predicting environmental concentrations (B.F. Lyon); considerations in selecting and developing multiple exposure pathway models (P.D. Jennings); the comparison of measured, modeled, and monitored dioxin concentrations with indirect multipathway exposure modeling results (P.J. Koval); and the assessment of health risks due to hazardous air pollutant emissions from electric utilities (C.L. French). This session also included a panel discussion.

The presentations in Session 4a covered the methods to detect developmental toxicants. Discussions included teratology - tools of the trade (K.K. Sulik); methods to detect developmental toxicants - ethylene glycol (R.W. Tyl); *in vitro* approaches to developmental toxicity testing (G.P. Daston); and methods for detecting long-term CNS dysfunction after prenatal exposure to neurotoxins (C.V. Vorhees).

The subject of Session 4b was pollution prevention and included the topics of Risk-Based Tiered Approach (RBTA) for pollution prevention (R.G. Elves); Armstrong Laboratory involvement in halon toxicity issues (A. Vinegar); developments in the application of impact assessment methodologies for pollution prevention (J.C. Bare); the U.S. Army's Health Hazard Assessment Program (W.M. McDevitt); toxicology in the Army Acquisition Pollution Prevention Program (J.A. Macko, Jr.); and integrating environment, safety, and health into the Air Force acquisition process (S.G. Forbes).

Session 5 dealt with trichloroethylene risk assessment. Topics included trichloroethylene health risk assessment (V.J. Cogliano); the epidemiology behind TCE (C. S. Scott); trichloroethylene noncancer toxicities - biologically based modeling and dosimetrically based risk assessment (H.A. Barton); pharmacokinetics and dosimetry of TCE (J.W. Fisher); dose response approaches for TCE carcinogenicity (L.R. Rhomberg); and communicating risk to the public - TCE as an example (K.M. Kurtz). A panel discussion also concluded this session.

Eight workshops on relevant topics were presented at the 1997 conference and were attended by many of the participants. Evening poster and database sessions provided additional scientific information exchange in an informal manner. There were 39 oral presentations, 67 poster presentations, three database presentations, and 274 participants in the three and one-half day conference. In a written critique provided for comments, the conference was highly rated by the participants. Proceedings of the conference were published in the scientific journal *Drug and Chemical Toxicology, 1997.*

9.2 1998 TRI-SERVICE TOXICOLOGY CONFERENCE ON ISSUES AND APPLICATIONS IN TOXICOLOGY AND RISK ASSESSMENT

L.A. Doncaster

Planning was initiated in May 1997 for the 1998 toxicology conference, "Conference on Issues and Applications in Toxicology and Risk Assessment". It will be held 27 through 30 April 1998 at the Hope Hotel and Conference Center at Wright-Patterson Air Force Base, OH. The conference will be sponsored by Tri-Service Toxicology, Wright-Patterson Air Force Base; the National Center for Environmental Assessment, United States Environmental Protection Agency; and the Division of Toxicology, Agency for Toxic Substances and Disease Registry. The planning committee for the conference includes representatives from each sponsoring agency and coordination is being handled by ManTech Environmental Technology, Inc., Toxic Hazards Research.

The goals of the conference are (1) exploration of new methodologies for inhalation toxicology and for determining exposure and effects in humans; (2) understanding jet fuel toxicity from an occupational and environmental perspective; and (3) examination of the issues and approaches for acute toxicity/exposure, emergency response situations, and multipathway exposure assessment.

The ManTech Environmental Work Plan, which designated a conference coordinator and described invitation and registration procedures, publication procedures, technical support, and the administration of continuing education credits, was submitted to and approved by the Contract Technical Monitor.

An initial conference announcement was placed in a major scientific journal and the conference announcement has been mailed to over 3100 individuals.

SECTION 10

RESEARCH SUPPORT

10.1 PATHOLOGY SUPPORT (NECROPSY, EMBEDDING, HISTOLOGY, AND ELECTRON MICROSCOPY)

J.R. Latendresse and M. Parish

Necropsy support was provided in accordance with protocol requirements, standard operating procedures, or as determined by the veterinary pathologists. Routine, accepted methods of anesthesia were followed for terminal bleeding or euthanasia of laboratory animals. Necropsy procedures included terminal sampling of blood, humane euthanasia of laboratory animals, determination of terminal body weights, detailed dissection, weighing of required organs, and collection and fixation of gross lesions and other required tissues for light microscopic examination.

Histologic processing of tissues included trimming, orientation of tissues in embedding cassettes, paraffin embedding, microtome sectioning of tissues to specified thickness, and applications for routine or special staining and cover slipping. Uniformly processed, high quality slides were prepared for reviewing by veterinary pathologists. A pathology specimen archive was maintained with controlled access as required by Good Laboratory Practices.

The histology/necropsy support technicians continued to implement their skills in tissue processing required for molecular pathology methods now being used extensively in the laboratory. These new tissue processing requirements included unique fixation protocols, sterile sectioning procedures, and microwave processing to enhance antigen, DNA, and RNA retrieval. The tissue staining methods were also unique. The stains were performed on a state-of-the art, computer-based automated stainer operated by laboratory personnel.

Processing of tissues for transmission electron microscopy included trimming, fixation, staining, dehydration, plastic embedding, microtomy and ultramicrotomy of tissues to specified thickness, and routine and advanced post-staining of tissue sections. Specimen processing for scanning electron microscopy and X-ray microanalysis included fixation, dehydration, critical-point drying, and gold sputtering or carbon coating. Specimens were examined on a JEOL 1200 TEM or AMRAY 1000B SEM, and resulting micrographs were reviewed by veterinary pathologists. This year, as a result of the very limited use and cost analysis, the electron microscopy laboratory was shut down. In the future, any ultrastructural assessment requirements will be subcontracted.

Necropsy and histology personnel continued to play a significant role in keeping our laboratories in compliance with the AFMC mandated hazardous material (HM) and hazardous waste (HW) control program. These personnel actively participated in managing HM and HW generated as a result of research activities, and helped facilitate the ongoing transfer of all HM to a centralized issue point (AFMC Pharmacy concept).

Pathology staff members processed the following research animals and provided the following research support during the past reporting period (Tables 10.1-1 through 10.1-3.).

TABLE 10.1-1. NECROPSY YEARLY REPORT - OCTOBER 1996 THROUGH SEPTEMBER 1997

Species	No. Animals
OCTOBER 1996	
RAT	44
G.PIG	17
RABBIT	2
FERRET	2
PIGEON	1
NOVEMBER 1996	
RAT	76
MICE	3
RABBIT	1
DECEMBER 1996	
RAT	193
MICE	2
RABBIT	28
G.PIG	11
JANUARY 1997	
RAT	39
MICE	6
RABBIT	10
G.PIG	1
FERRET	2
FEBRUARY 1997	
RAT	38
MICE	29
HAMPSTER	1
MARCH 1997	
RAT	96
MICE	3
G.PIG	2
APRIL 1997	
RAT	33
MICE	3
G.PIG	4
MAY 1997	
RAT	27
MICE	8
G.PIG	2
JUNE 1997	
RAT	97
MICE	7
JULY 1997	
RAT	208
AUGUST 1997	
RAT	44
MICE	2
SEPTEMBER 1997	
RAT	75
MICE	2

Species	No. Animals
Rat	970
Mice	65
G. Pig	37
Rabbit	41
Ferret	4
Pigeon	1
Hamster	1
Totals	1119

TABLE 10.1-2. EMBEDDING YEARLY REPORT - OCTOBER 1996 THROUGH SEPTEMBER 1997

Species	Cases	Blocks
OCTOBER 1996		
RAT	11	56
G.PIG	12	12
RABBIT	2	2
FERRET	1	11
PIGEON	1	11
NOVEMBER 1996		
RAT	90	393
MICE	4	12
RABBIT	2	15
FERRET	2	11
SPECIAL	5	5
DECEMBER 1996		
RAT	193	430
MICE	2	10
RABBIT	7	15
G.PIG	16	20
SPECIAL	2	5
JANUARY 1997		
RAT	48	108
MICE	6	18
RABBIT	40	140
G.PIG	4	15
FERRET	1	15
DOG	1	1
SPECIAL	1	1
FEBRUARY 1997		
RAT	11	25
MICE	6	42
FERRET	1	17
MARCH 1997		
RAT	26	250
MICE	25	21
G.PIG	3	32
HAMSTER	1	5
APRIL 1997		
RAT	65	766
MICE	3	9
G.PIG	2	19
SPECIAL	1	4
MAY 1997		
RAT	28	73
MICE	8	24
G.PIG	4	36
SPECIAL	2	4
RECUT	1	1
JUNE 1997		
RAT	11	27
MICE	7	21
FERRET	1	7
JULY 1997		
RAT	13	36
AUGUST 1997		
RAT	38	312
MICE	1	3
RECUT	1	1
SEPTEMBER 1997		
RAT	109	380

Species	Cases	Blocks
Rat	534	2856
Mice	62	172
G. Pig	41	134
Rabbit	51	172
Ferret	6	61
Pigeon	1	11
Dog	1	1
Hamster	3	32
Special	11	19
Recut	2	2
Totals	712	3460

TABLE 10.1-3. HISTOLOGY YEARLY REPORT - OCTOBER 1996 THROUGH SEPTEMBER 1997

Species	Cases	Blocks	Slides
OCTOBER 1996			
RATS	18	66	68
RABBITS	2	2	2
FERRETS	1	11	11
PIGEON	1	11	13
CAT	1	1	2
G.PIG	12	12	24
NOVEMBER 1996			
RATS	64	282	282
MICE	5	16	16
DOG	4	5	11
DECEMBER 1996			
RATS	97	117	205
MICE	1	7	7
RABBITS	3	11	31
G.PIG	6	6	6
JANUARY 1997			
RATS	173	272	375
MICE	6	18	18
RABBITS	32	243	529
G.PIG	2	8	8
FEBRUARY 1997			
RATS	17	39	39
MICE	28	64	130
FERRETS	3	25	29
G.PIG	14	14	14
HAMSTERS	5	5	5
MARCH 1997			
RATS	46	619	619
MICE	3	9	9
G.PIG	11	36	36
APRIL 1997			
RATS	117	909	1134
MICE	1	3	3
G.PIG	1	10	10
MAY 1997			
RATS	53	151	223
DOG	1	1	1
G.PIG	3	30	30
JUNE 1997			
RATS	14	36	36
JULY 1997			
RATS	12	35	35
AUGUST 1997			
RATS	21	46	46
SEPTEMBER 1997			
RATS	54	418	418

Species	Cases	Blocks	Slides
Rat	686	2990	3480
Mice	46	123	189
Rabbits	37	256	562
Ferrets	5	43	47
Dog	5	6	11
Pigeon	1	11	13
G. Pig	49	116	128
Hamster	5	5	5
Csy	1	1	2
Specials	6	14	20
Totals	836	3559	4446

10.2 QUALITY ASSURANCE

M.G. Schneider

PERSONNEL ACTIVITIES

The Quality Assurance Coordinator was elected to a two-year term as a Director of the Mid West Regional Chapter of the Society of Quality Assurance and attended two Regional Chapter meetings where continuing training in GLPs was received. The Quality Assurance Associate participated on the program management team during the Toxicology Conference on Issues and Applications in Toxicology and Risk Assessment.

SOP ACTIVITIES

The Quality Assurance Associate reviewed, prepared-for-approval, distributed, and placed on the Division LAN system sixty-six new or revised Standard Operating Procedures.

TECHNICAL EDITING

The Quality Assurance Associate was principal editor for the 1996 THRU Annual Report, and performed technical editing on the following reports:

- "A Physiologically Based Pharmacokinetic Model for Chloral Hydrate and its Metabolites, Trichloroethanol, Trichloroacetic Acid and Dichloroacetic Acid in B6C3FI Mice." R. Abbas, et al.
- "A Physiologically Based Pharmacokinetic Model for Trichloroethylene and its Metabolites, Chloral Hydrate, Trichloroacetate, Dichloroacetate, Trichloroethanol, and Trichloroethanol Glucuronide in B6C3FI Mice." R. Abbas and J. W. Fisher.
- "Developmental Toxicity Screen of Ammonium Dinitramide Using *Hydra Attenuata.*" R. E. Wolfe, et al.
- "Electrospray Analysis of Biological Samples for Trace Amounts of Trichloroacetic Acid, Dichloroacetic Acid, and Monochloroacetic Acid." W. T. Brashear, et al.
- "Physiologically Based Pharmacokinetic Models for Trichloroethylene and its Oxidative Metabolites in Mice and Humans." J. W. Fisher.
- "Effects of Ammonium Dinitramide on Preimplantation Embryos in Sprague-Dawley Rats and B6C3FI Mice." L. J. Graeter, et al.
- "Developmental Toxicity Screen of Ammonium Perchlorate Using *Hydra Attenuata.*" P. D. Confer, et al.

- "PBPK Modeling of Short-Term (O to 5 Minutes) Human Inhalation Exposures to Halogenated Hydrocarbons." A. Vinegar and G. W. Jepson
- "An Electron Capture Gas Chromatography Method for Analysis of Dichloroacetic Acid, Trichloroacetic Acid, and Trichloroethanol in Biological Matrices." H. M. Zhang, et al.
- "Histological and Immunohistochemical Differences in DCA- and TCA-Promoted Mouse Hepatocytic Foci and Neoplasms," J. R. Latendresse and M. A. Pereira.
- "Acute Oral Toxicity Evaluation and Genotoxicity Testing of Hexakis (2,2,2-trifluoroethoxy) Cyclotriphosphazene, a Replacement Candidate for Ozone Depleting Substances." R. E. Wolfe, et al.
- "Developmental Toxicity Screen of Diethyleneglycol Monomethylether Using *Hydra Attenuata.*" R. E. Wolfe.
- "Developmental Toxicity Screen of 1,3,5-Trinitrobenzene Using *Hydra Attenuata.*" R. E. Wolfe, et al.
- "Acute Inhalation Toxicity Evaluation of a 9:1 Mixture of 1, 1, 1,3,3,3 -Hexafluoropropane and I -Bromopropane, a Replacement Candidate for Ozone Depleting Substances." R. E. Wolfe, et al.
- "Acute Toxicity Evaluation of a new Non-Corrosive Decontamination Solution." R. E. Wolfe, et al.
- "Physiologically Based Pharmacodynamic Modeling of Chemically Induced Oxidative Stress." J.Z. Byczkowski, et al.
- "Simulated Blood Levels of CF31 in Personnel Exposed During Its Release From an F-15 Jet Engine Nacelle and During Intentional Inhalation." A. Vinegar, et al.
- "Acute and Subchronic Toxicity Evaluation of the Halon Replacement Candidate Phosphorus Tribromide." R. E. Wolfe, et al.
- "Inhalation of Halon 1301, HFC-134a, and HFC-277ea by Humans." A. Vinegar, et al.

10.3 HEALTH & SAFETY

M.G. Schneider

GENERAL

The Health and Safety Representative provided occupational health and safety support for ManTech staff and was the Unit Safety Representative for AL/OET, Toxicology Division.

TRAINING

ManTech staff who worked in laboratory areas received annual training in Wright-Patterson Air Force Base hazardous materials/hazardous waste (HM/HW) management policies and procedures, and radiation safety policies and procedures, the OSHA Laboratory Chemical Standard, and the OSHA Bloodborne Pathogen Standard. ManTech staff who were designated as hazardous waste initial accumulation point managers received annual training provided by WPAFB Environmental Management for Environmental Protection Agency Resource Conservation Recovery Act (RCRA) hazardous waste and biannual training on Department of Transportation (DOT) hazardous materials transportation regulations. The Health and Safety Representative provided safety orientation training for all new staff (DOD and Contractor) assigned to the Toxicology Division. The Health and Safety Representative received special training in explosive safety, radiation safety, EM hazardous waste regulations (RCRA 8-hour), DOT hazardous material regulations, EM hazardous materials management system (DM-HMMS), and Animal care and use requirements for investigators. The Health and Safety Representative completed the annual review and revisions of the Toxicology Division Chemical Hygiene Plan and Bloodborne Pathogen Exposure Control Plan to comply with OSHA and Air Force regulations.

EXPLOSIVES SAFETY

The Health and Safety Representative continued as alternate for the AL/OET explosives safety program. Originally each explosive item stored in the facility required licensing by WPAFB. The Health and Safety Representative assisted Base Explosive Safety with the identification of explosive class materials whose use by OET could be classified as laboratory use only. This allowed their use without the licensing and storage requirements. This gave investigators ready access to the materials for toxicity testing purposes. The Health and Safety Representative assisted in the training of the investigators and technicians using explosive materials.

PERSONNEL SAFETY

ManTech staff were provided with appropriate safety equipment as required by specific work assignments. Purchase agreements were established with local vendors for safety glasses and safety shoes. The medical surveillance program, which included employee physicals (pre-employment and exit), follow-up examinations on work-related injury or illness for work release, consultation for pregnant staff or suspected work-related illness, and initiation of Workers Compensation paperwork was continued. Annual physicals for staff working in laboratories and with animals were provided by Air Force Occupational Medicine. Hepatitis B immunization was offered to staff who might encounter bloodborne pathogens in their jobs.

The Health and Safety Representative coordinated the AL/OET respiratory protection program. DOD personnel were trained and fit tested for respirator wear by Base Occupational Health, while Contractor staff were trained and fit tested by the Health and Safety Representative..

INSPECTIONS

Work areas occupied by ManTech staff were subject to annual inspections by Air Force safety personnel from Human Systems Center, Brooks AFB, WPAFB (Environmental Management, Base Explosive Safety, Public Health), and Armstrong Laboratory North. Specific programs as Environmental Compliance and Management Program, Radiation Safety, Hazardous Waste (chemical and biological), and Explosives Safety were inspected several times during the year. The Health and Safety Representative accompanied these inspection teams. Response by ManTech to the findings in its areas of responsibility was always timely. The Health and Safety Representative also conducted spot and monthly safety inspections, including testing eyewashes and showers. The Health and Safety Representative inspected laboratory spill kits semiannually. Issues of immediate concern were addressed with staff and supervisors on-the-spot. Work requests were submitted through the OET Building Superintendent to correct building-related safety issues.

10.4 COMPUTER SUPPORT

M.J. Walsh

Network

In Fiscal Year 1997, the ManTech team upgraded the Network topology of the entire building. The staff undertook the project of running 10Base-T, Category five wiring throughout building 79. Four Baystack 102 hubs were installed, three Baystack 201 10/100 hubs, and a Bay Networks 10/100 switch. This replaces the Thin-net, coaxial cable that the network had been running on. All of the servers were put onto a separate port on the switch. This enhanced the speed of server access for all users. Optivity for Windows was installed on a dedicated machine to manage the network topology (hubs and switch). Six of the seven Digital Equipment Corporation Terminal servers were removed from service. The two Digital Equipment Multi-Port Repeaters (DEMPRs) used by the coaxial cable plant were unplugged.

After the main NetWare server, Mariner was put on a 100Mb/s connection, several users were unable to connect to the Network. These users were on the old coaxial cable plant, which only had one collusion domain. Once these users were put onto the Twisted pair cable, which was segmented, the problems went away. Once the cabling upgrade was complete, there was still a problem with certain Network cards having trouble connecting. The Standard Microsystems Corporation Network cards do not work well on a busy collusion domain. Many of these cards were replaced. The ones that remained in service were put onto relatively quiet Network segments.

All of the building's printers were attached to the Network so that all users could access them. A dynamic host configuration protocol was set up on the servers to automatically hand out and manage internet protocol addresses. A NetWare server was set up to run NetWare Connect, and a modem pool of four modems was attached to it for Dial in-dial out capabilities. All servers were attached to universal power supplies. The NetWare servers were upgraded to version 4.11. Cheyenne ARCServe was installed to perform backups. This replaces the Palindrome software. Two Microsoft NT servers were installed for WEB and Internet activities.

The Toxicology Division owns approximately 85 Government Technology Systems Inc. computers. These were all upgraded in 1997. The computer support team replaced the central processing unit chip with a Pentium 83 chip. Most machines in building 79 were upgraded to Windows 95. A batch script install was developed to accomplish this. A batch script install to upgrade all machines to run Office 95 was developed and executed on all machines. McAfee virus-scan software was installed on all machines,

and a process to update the virus signature files on a monthly basis was implemented. Many computers in the labs have to run Windows 3.1, because they also control research equipment. A network card was installed in most of these machines, and the Novell 32-bit client for Disk Operating System/Windows was installed. This enabled the researchers to copy the data to the Network, and then access it from their office machines for analysis. This eliminated the need to run back and forth with floppy diskettes.

The computer support team faced quite a few challenges in FY 1997. There was quite a bit of turnover in personnel. Mr. Dave Derry and Mr. Steve Myers performed most of the upgrades detailed above. In May, Mr. Myers left his position. In July, Mr. Derry left his position and was replaced by Ms. Maggie Brudnicki. In August, Mr. Mark MacLean filled the open position.

Technical challenges included widespread-computer viruses that were eventually contained. There was an internetwork packet exchange communication problem where an internetwork packet exchange driver on the server became corrupted and resulted in intermittent communications with other parts of the Armstrong Laboratory NetWare Tree. The resolution was to restart the servers. The Pentium 83 Overdrive process would not work in the "Energy Star" model Government Technology Systems Inc. Desktop IV computers.

Application Development

During Fiscal Year 1997, several database issues were addressed. One issue was to develop a database in order to track equipment, supplies, and expenditures. At first, several databases were created by Ms. Alicia Howard (CSAM, ESAM, PUBSAM), but those databases did not fulfill the needs of the client. Ms. Jennifer Benincasa began to work on a single database called the Operational Toxicology Management Database (OTMD) to encompass all of the required areas. Most of the year was used for the development of OTMD and its help documentation. The system will be used beginning in Fiscal Year 1998. It was decided to track equipment in a separate database, other than the OTMD. A simple database was created in Access to be used by SSgt Wes Nelson.

The pathology department's need for a database to be used for data entry was also completed. The MatLitterExam.mdb database was used to enter maternal necropsy and litter examinations. Maj Jeff Eggers, Dr. John Latendresse, and Dr. Linda Graeter worked with Ms. Jennifer Benincasa to develop this database.

The last issue concerned the historical PathTox data stored on the virtual address extension. Originally, Ms. Jennifer Benincasa was to create a database that could be used like PathTox, but in a Windows format and would run from the structured query language server. After much debate, it was concluded

that re-creating a windows-based application to enter and retrieve PathTox data would be too complex and would take more time, manhours, and resources to be beneficial. Due to the large cost associated with running the virtual address extension and the complexity of the data, the task of creating a solution was handed over to the Network Administrators, Mr. Dave Derry and Mr. Steve Myers, for their evaluation.

WWW Site Development

During Fiscal Year 1997, most of the WWW Site work was completed by Mr. Steve Myers. He defined the needs and goals of the web site, to be implemented with a four-step plan (site design, Java and CGI scripting, implementation of forms/production site). He worked with AL//OET management throughout development and implemented his plan to create the WWW Site for Armstrong Lab. A Windows NT 4.0 Server and a Microsoft Internet Information Server were installed on VOYAGER. The Microsoft Internet Information Server (IIS) on VIKING was configured as the new intranet server. Intranet server was installed on a machine other than Internet (VOYAGER) for enhanced security of internal data and increased performance of both services. Microsoft structured query language server was selected as our database back-end because of its tight integration with the Windows NT operating system and the System Management Server (SMS) in the BackOffice suite. Ms. Jennifer Benincasa took over the roll for maintaining the WWW Site after Mr. Steve Meyers left in the last quarter of 1997. She continued to update the Text version, add new documents, including recent publications, and converted SOT abstracts and Tox Conference agendas to portable document file (.pdf) format.

Planned Activities for Fiscal Year 1998

Application Development

The OTMD database will be used to track costs and order supplies. Many enhancements will be implemented as they are required or requested by users. Any problems with the system will be fixed as soon as they are found. Other Access databases can be created for users if necessary.

WWW Site Development

The current AL WWW Site needs to be upgraded into a new format. AFRL has produced new WWW Site standards. These standards include, for example, no-frames, change of graphics, fonts, and required content. Ms. Jennifer Benincasa, in cooperation with the client, will make all the necessary changes to convert the WWW Site in its new required format.

SECTION 11

APPENDIX

APPENDIX

PRODUCTS LIST FOR 1997

1 October 1996 - 30 September 1997

AWARDS and HONORS

Byczkowski, J.Z., Men of Achievement, 1996-1997. 17th Edition, International Biographical Centre, Cambridge, UK.

Byczkowski, J.Z., Who's Who in the World, 1997. 14th Edition, *1997 Marquis Who's Who*, New Providence, NJ.

Byczkowski, J.Z., Who's Who in America, 1997. 52nd Edition, *1997 Marquis Who's Who*, New Providence, NJ.

Byczkowski, J.Z., Who's Who in the Midwest, 1996-1997. 25th Edition, Marquis Who's Who, New Providence, NJ.

Byczkowski, J.Z., Who's Who in Polish America, 1996-1997. 1st Edition, Bicentennial Publishing Corporation, New York, NY.

Dodd, D.E., Serving as member of the Editorial Board of *Drug and Chemical Toxicology*, 1997 to the present.

Dodd, D.E., Served as member of the Editorial Board of *Inhalation Toxicology,* 1995 to the present.

Dodd, D.E., Elected President-Elect of the Ohio Valley Regional Chapter of the Society of Toxicology, 1996 to the present.

Doncaster, L.A., Recipient of ManTech Environmental's President's Award for Excellence, 1996.

Garrett, C.M., (with Lipscomb, J.C. and Snawder, J.E.), Scientific Achievement Award in recognition of publication, "Cytochrome P450-Dependent Metabolism of Trichloroethylene: Interindividual Differences in Humans", presented 14 April 1997.

Schneider, M.G., Served as member of the Nominating Committee for the Society of Quality Assurance for 1997.

Schneider, M.G., Serving as a Director on the Board of the Midwest Regional Chapter of the Society of Quality Assurance for a three-year term.

JOURNAL PUBLICATIONS

Barton, H.A., and S. Das. 1996. Alternatives for a Risk Assessment on Chronic Noncancer Effects from Oral Exposure to Trichloroethylene. *Regulatory Tox & Pharmacology,* **24**:269-285.

Brashear, W.T., C.T. Bishop, and **R. Abbas.** 1997. Electrospray Analysis of Biological Samples for Trace Amounts of Trichloroacetic Acid, Dichloroacetic Acid, and Monochloroacetic Acid. *Analytical Toxicology,* **21**:330-334.

Byczkowski, J.Z. and A.P. Kulkarni. 1997. Oxidative Stress and Pro-oxidant Biological Effects of Vanadium. *Vanadium in the Environment,* Part 2: Health Effects, Chapter 12 (Advances in Environmental Sciences and Technology, Nriagu, J.O. Ed), pp. 355-391.

Byczkowski, J.Z., S.R. Channel, T.L. Pravecek, and C.R. Miller. 1996. Mathematical Model for Chemically Induced Lipid Peroxidation in Precision-Cut Liver Slices: Computer Simulation and Experimental Calibration. *Computer Methods and Program in Biomedicine,* **50**:73-84.

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Dodd, D.E., E.R. Kinkead, R.E. Wolfe, H.F. Leahy, J.H. English, and **A. Vinegar.** 1997. Acute and Subchronic Inhalation Studies on Trifluoroiodomethane Vapor in Fischer 344 Rats. *Fundament. Appl. Toxicol.,* **35**:64-77, AL/OE-JA-1996-0096.

Dodd, D.E., A.D. Ledbetter, and A.D. Mitchell. 1997. Genotoxicity Testing of the Halon Replacement Candidates Trifluoroiodomethane (CF3I) and 1,1,1,2,3,3,3-Heptafluoropropane (HFC-227ea) Using the Salmonella Typhimurium and L5178Y Mouse Lymphoma Mutation Assays and the Mouse Micronucleus Test. *Inhalation Toxicology,* **9**:111-131, AL/OE-JA-1996-0101.

Fisher, J.W., **D.A. Mahle,** L. Bankstron, **R. Greene,** and J. Gearhart. 1997. Lactational Transfer of Volatile Chemicals In Breast Milk. *Am. Ind. Hyg. J.* **58**:425-431.

Jepson, G.W. and J.N. McDougal. 1997. Physiologically Based Modeling of Nonsteady State Dermal Absorption of Halogenated Methanes from an Aqueous Solution. *Toxicol. Appl. Pharmacol.*, **144**:315-324.

Latendresse, J.R. and M.A. Pereira. 1997. Dissimilar Characteristics of *N*-Methyl-*N*-Nitrosourea-Initiated Foci and Tumors Promoted by Dichloroacetic Acid or Trichloroacetic Acid in the Liver of Female B6C3F1 Mice. *Toxicologic Pathology*, **25**:(5)433-440.

Lipscomb, J.C., **C.M. Garrett**, and J.E. Snawder. 1997. Cytochrome P450-Dependent Metabolism of Trichloroethylene: Interindividual Differences in Humans. *Toxicology and Applied Pharmacology*, **142**(2):311-8.

Lipscomb, J.C., **G.W. Buttler, and P.D. Confer.** 1997. Chloral Hydrate Formation in the Japanese Medaka Minnow. *Annals of Clinical Lab. Sci.*, **27**:158-163.

Lipscomb, J.C., **D.A. Mahle, W.T. Brashear, and C.M. Garrett.** 1996. A Species Comparison of Chloral Hydrate Metabolism in Blood and Liver. *Biochem. Biophys. Res. Commun.* **227**(2):340-350.

McDougal, J.N., J. Grabau, L. Dong, D.R. Mattie, **and G.W. Jepson.** 1997. Inflammatory Damage to Skin by Prolonged Contact with 1,2-dichlorobenzene and Chloropentafluorobenzene. *Microscopy Research Techniques*, **37**(3): 214-20.

Reo, N.V., **L. Narayanan**, K.B. Kling, and M. Adinehzadeh. 1996. Perfluorodecanoic Acid, a Peroxisome Proliferator, Activates Phospholipase C, inhibits CTP: phosphocholine Cytitdylyltransferase, and elevates Diacylglycerol in Rat Liver. *Toxicology Letters*, **86**:1-11.

Smith, M.A., M.C. Gothaus, D.A. Warren, and **J.R. Latendresse**. 1997. Automated Procedure for In Situ Detection of Apoptosis. *The Journal of Histotechnology*, **20**:(4)329-335.

Vinegar, A., and G.W. Jepson. 1996. Cardiac Sensitization Thresholds of Halon Replacement Chemicals Predicted by Physiologically-based Pharmacokinetic Modeling. *Fund. Appl. Toxicol.* **30**:(1)(Pt 2):292.

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Vinegar, A., G.W. Jepson, and J.H. Overton. 1997. PBPK Modeling of Short-Term (0 to 5 Minute) Human Inhalation Exposures to Halogenated Hydrocarbons. *Inhalation Toxicology,* **36**:1 (pt 2).

Yu, K.O., D.E. Tillitt, **J.Z. Byczkowski,** G.A. Burton, S.R. Channel, **J.M. Drerup, C.D. Flemming,** and J.W. Fisher. 1996. *In vivo/In vitro* Comparison of the Pharmacokinetics and Pharmacodynamics of 3,3',4,4-tetrachlorobiphenyl (PCB77). *Toxicology and Applied Pharmacology,* **141**:434-438.

Yu, K.O., **J.Z. Byczkowski, J.M. Drerup,** J.D. McCafferty, and J.W. Fisher. 1996. Pharmacokinetics and Pharmacodynamics of 3,3',4,4'-tetrachlorobiphenyl in the Rats: PBPK Model Development. 35th Annual Meeting of the Society of Toxicology, Anaheim, CA. *The Toxicologist* **36**: 247 (1266).

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Byczkowski, J.Z., C.D. Flemming, M.A. Curran, C.R. Miller, W.J. Schmidt, A.P. Moghaddam, and S.R. Channel. 1997. "Physiologically Based Pharmacodynamic Modeling of Chemically Induced Oxidative Stress" AL/OE-TR-1997-0130.

Caracci, M.C., R.S. Geary, C.M. Wall, **and G.W. Jepson.** 1995. "Development of Microdialysis Probe Method for Partition Coefficient Determination for Pharmacokinetic Modeling" AL/OE-TR-1995-0090.

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Creech, J.R., R.K. Black, B.L. Garrity, R.J. Williams, **J.N. McDougal, G.W. Jepson, R. Abbas, L. Dong, and A. Vinegar.** 1995. "Inhalation Uptake and Metabolism of Iodohalogenated Compounds, CF3I, C6F13I, and C3F7I" AL/OE-TR-1995-0089.

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